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PRINCIPAL INVESTIGATOR: Sandra J. Gendler, Ph.D.

CONTRACTING ORGANIZATION: Mayo Clinic Scottsdale
Scottsdale, Arizona 85259

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Sandra J. Gendler, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mayo Clinic Scottsdale
Scottsdale, Arizona 85259

E-Mail: gendler.sandra@mayo.edu

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13. ABSTRACT (Maximum 200 Words)

This project is focused on the development of novel tumor vaccines directed at MUC1 and other tumor antigens. Our specific aims are: 1) To assess the effectiveness of vaccine formulations against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice and 2) To translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden. The model of spontaneous mammary cancer is the MUC1-expressing polyoma middle T antigen mice (MMT). We have tested four vaccines in the preclinical mouse model: 1) liposomal MUC1 tandem repeat peptide, 2) dendritic cells (DCs) pulsed with tumor lysate, 3) DCs fused to MMT tumor cells, and 4) adoptive transfer of MUC1-specific cytotoxic T lymphocytes (CTLs). All vaccines elicited a strong immunological response, although CTLs in the tumor environment were tolerized. DCs pulsed with lysate accompanied by co-stimulation (4-1BB) greatly reduced tumor burden and reduced tolerance in MMT mice. Aim 2, to develop a clinical trial, is underway. The clinical trial protocol is under review. It is a phase I trial testing MUC1 and HER-2/neu class I and class II peptides in breast cancer patients free of disease.

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INTRODUCTION

This project is focused on the development of novel tumor vaccines directed at MUC1 and other tumor antigens. MUC1 is expressed on most breast cancers and often elicits cellular and humoral immune responses in humans. However, these responses are not sufficiently strong to eradicate tumors. MUC1 is a candidate peptide for novel immunotherapy strategies to strongly activate the immune system to eradicate tumors expressing these epitopes. In tumors, there is strong over expression of MUC1 on tumor cells and in circulation, expression is no longer restricted to the apical domain of cells, and glycosylation is altered, revealing immunodominant tumor-specific peptide sequences.

In our preclinical studies we have utilized mice that develop spontaneous mammary gland cancer that express MUC1. MUC1 transgenic mice (MUC1.Tg) were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice (1, 2). Mice transgenic for this protein develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene. All mice are congenic on the C57BL/6 background to eliminate strain-specific modifier effects. In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) (2). Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas in 100% of the female mice. The model is described in detail in the paper supplied in the appendix (3). The MMT mouse appears to be an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity and auto-immunity to MUC1 as mammary tumors develop spontaneously.

The **hypothesis** of our study is that enhancing MUC1-specific immunity will result in anti-tumor immunity. We propose to develop an optimal cancer vaccine using epithelial cell mucin MUC1 peptides or protein or MUC1-expressing tumors presented by DCs as immunogen. The most successful therapies will be tested in phase I clinical trials. An Additional hypothesis is that tolerance occurs within the tumor environment, although immunization strategies can be developed to overcome tolerance.

Our **specific aims** are: **1)** to assess the effectiveness of vaccine formulations against MUC1 in the prevention and treatment of spontaneous breast carcinomas in mice and **2)** to translate the most effective vaccine strategies into phase I clinical trials in patients with minimal residual disease. The draft clinical trial protocol for aim 2 is included in this annual report, although it has not yet received IRB or FDA approval nor been formally submitted for DOD approval.

RESULTS (BODY)

Specific Aim 1: To assess the effectiveness of vaccine formulations against MUC1 and other tumor antigens in the prevention and treatment of spontaneous breast carcinomas in mice.

We reported last year that dendritic cells pulsed with tumor lysates were an effective immunization strategy that completely protected mice from subsequent challenge with injected tumor cells. The challenge is to achieve effective results in the spontaneous mammary tumor MMT mice.

Immunotherapy with dendritic cells pulsed with tumor lysate in MMT mice
MMT mice were immunized with DC pulsed with MMT whole tumor lysate starting at 3 weeks of age; treatment was continued every three weeks until mice were about 20 weeks of age. Mice were injected every three weeks with 1 x 10^7 DCs pulsed with 20 ug/ml lysate (overnight pulsation followed by maturation with LPS at 1 ug/ml for 24 hours). There was no significant difference in tumor burden (data not shown). However, all of the immunized mice developed highly lytic (85-100% lytic activity) MUC1-specific CTLs as measures by a standard ^{51}Cr-release assay against B16 melanoma cells expressing MUC1 (Figure 1). By specific IFN-γ ELISPOT assay, T cells from immunized MMT mice showed significantly higher IFN-γ production (150-250 spots per 2 x 10^5 T cells) as compared to untreated MMT mice (<50 spots per 2 x 10^5 T cells) (Figure 2). Thus, once again our data indicate that in a spontaneous model of breast cancer, a robust MUC1-specific cellular immune response does not lead to a robust clinical response. Since we know that the tumor microenvironment releases factors that down regulate CTL activity (Annual Report 2002), we determined if the TILs in immunized mice were tolerant to MUC1 and if they were capable of IFN-γ production. We found that the $CD4^+$ T cells in immunized mice were highly proliferative in response to MUC1 antigen presented on autologous APCs (12269 cpms in immunized mice versus 3675 cpms in control mice). On the other hand, the $CD8^+$ T cells from immunized mice showed low proliferation (6000 cpms) in response to MUC1 antigen as compared to controls (4000 cpms). Nevertheless, both the $CD8^+$ and the $CD4^+$ T cells isolated from DCL treated MMT tumors were capable of producing IFN-γ (for CD4 cells: ~130 spots/1 x 10^5 cells in immunized mice compared to ~10 spots/1 x 10^5 cells in control MMT mice; for CD8 cells: ~88 spots/1 x 10^5 cells in immunized mice compared to ~14 spots/1 x 10^5 cells). This suggests that proliferation to MUC1 antigen may be a more sensitive assay to determine tolerance than the IFN-γ production. These preliminary data suggested that some type of co-stimulation would be required to enhance the $CD8^+$ CTL activity and counter the induction of tolerance.

Tolerance and signaling through OX40 (CD134) Tolerance is brought about by failure of the T cells to efficiently expand to antigen, and by the surviving cells entering into a state of hypo-responsiveness to subsequent antigen encounter. Although tolerance minimizes autoreactivity, it represents a serious problem in diseases such as cancer, where a lack of a T cell response can prevent immunity. Co-stimulatory receptors are known to prevent tolerance induction before it has fully developed and hence they are promising targets for therapeutic treatment. A member of the tumor-necrosis-factor receptor (TNFR) family, OX40 has recently been shown to be a primary co-stimulator of T cells that have encountered antigen rather than naïve T cells. OX40 is not constitutively present on naïve T cells, it peaks in expression 3-4 days after the initial activating signal and is rapidly and highly re-expressed on effector T cells (4-6). Using anti-OX40 as well as OX40-deficient mice, it has been shown that OX40 signals regulate the ability of $CD4^+$ T cells to accumulate in numbers, survive over time and promote effective help to $CD8^+$ cells and generate memory T cells (6). In addition, OX40 engagement can partially prevent

super-antigen induced T cell deletion (7). Because of its downstream role in late primary and secondary T cell responses, OX40 signals might be effective in reversing established tolerance. Hence, targeting OX40 might be ideal for modulating the T cell function of our experimental mice where T cells are tolerized within the tumor microenvironment.

OX40 Co-stimulation in MMT mice

We immunized three-week old MMT mice with 1 x 10^7 lysate fed DCs. Two days post primary immunization, OX40 antibody was injected i.p at 100ug/mouse. As controls, mice were treated with isotype-matched control IgG antibody. Immunizations were repeated every 3 weeks until termination of experiment. The treatment arms included: 1) tumor lysate-pulsed mature DC (DCL), 2) DCL + OX40 antibody, 3) DCL + control IgG, 4) OX40 antibody, 5) DC alone, and 5) saline treated. We obtained OX40 antibody from Dr. Michael Croft, which was tested for its effectiveness against previously tolerized MUC1-specific CTLs. Tolerized CTLs were grown in the presence of 2μg/ml OX40 antibody for 48 hours and proliferation to DCs pulsed with MUC1 peptide examined. Incubation with OX40 resulted in proliferation that was substantially greater than the proliferation of the tolerized CTLs (data not shown). At time of sacrifice, TDLN and mammary tissues were dissected. The $CD4^+$ and $CD8^+$ cells were sorted by magnetic or fluorescent activated cell sorter (MACS or FACS) and functional assays were performed. We compared the immune responses that developed during treatment and used tumor onset and tumor burden as the endpoints for determining the clinical effectiveness of the vaccine. All mice were sacrificed by week 19. There was no difference in tumor burden in treated versus control mice (data not shown).

Evaluation of the immune response in immunized mice: We observed that DCL, DCL + OX40 and DCL + IgG treatment significantly ($p < 0.001$) enhanced the ability of purified $CD4^+$ T lymphocytes isolated from tumor draining lymph node (TDLN) and stimulation with DCs pulsed with tumor lysate to produce IFN-γ by ELISPOT as compared to saline, OX40 and DC alone groups (~ 200 spots versus 10 - 30spots, Figure 3). Although OX40 treatment did not improve the $CD4^+$ T cell response when given in combination with DCL, it significantly increased the number of IFN-γ spot producing $CD8^+$ T cells when given in combination with DCL (~200 spots for DCL + OX40 versus ~60 spots for DCL; $p<0.001$). These results correlated with enhanced proliferation of $CD8^+$ T cells from TDLN in the DCL + OX40 treated mice (data not shown). Thus, OX40 costimulation was able to greatly amplify the $CD8^+$ T cell effector function with regards to IFN-γ production and proliferation in the TDLN of DCL + OX40 treated mice. Upon further evaluation of T cells isolated from within the tumor environment (TILs), we observed little to no proliferation and minimal numbers of IFN-γ spot forming cells (data not shown), strongly indicating that OX40 co-stimulation did not lead to reversal of T cell tolerance within the tumor microenvironment but was efficient in activating the T cells in the peripheral TDLN (Figures 3 and 4). Furthermore, these $CD8^+$ T cells from DCL + OX40 immunized mice did not induce MMT tumor cell killing *in vitro*, neither did they stain for perforin or granzyme (data not shown), both proteins essential for causing tumor cell lysis, once again suggesting that the $CD8^+$ T cells are non-cytolytic within the tumor microenvironment.

4-1BB agonistic antibody is effectively prevents the development of tolerance in MMT mice

We showed previously that MUC1-specific CTLs injected into the MMT tumor-bearing mice were anergized within the tumor environment within three weeks following injection. The sorted

TILs, recovered from the tumor microenvironment, exhibited reduced proliferation in response to MUC1 presented on irradiated DC and had reduced lytic capabilities, whereas the same cells that had not encountered tumor cells and were maintained in tissue culture remained highly cytolytic (Fig. 15A-C Annual Report 2002). 4-1BB (CD137) is an inducible T cell molecule belonging to the TNF receptor superfamily (8, 9). It has been previously reported that the anti-tumor CTL response is enhanced by agonistic anti-4-1BB mAb administration and is effective in overcoming T cell tolerance in in vitro models. A pilot study was performed in which MUC1-specific CTLs were adoptively transferred into MMT mice at 9, 12, and 15 weeks (2×10^7 cells per mouse intravenously). 4-1BB agonistic antibody (100 ug per mouse intraperitoneal) was injected weekly starting at week 10 and ending at week 16 and mice were sacrificed at 17 weeks. Tumors were removed and the tumor infiltrating lymphocytes (TILs) were isolated and sorted for the infused $CD8^+$ CTL clone based on their CD8 expression and T cell receptor (TCR) Vβ5 expression. Tumor burden was greatly decreased in both CTL treated and CTL plus 4-1BB treated mice compared to untreated mice (Figure 5). Figure 6 shows the photographic image of the dissected tumors, which clearly demonstrates an effect of both CTL and CTL plus the co-stimulator 4-1BB antibody. The sorted $V\beta5/CD8^+$ cells that were treated with 4-1BB antibody showed increased proliferation in response to MUC1 presented on irradiated DC compared to CTLs without the agonistic antibody, suggesting that co-stimulation helped to overcome the anergy induced in the tumor environment (Figure 7). Even more striking was the ability of the sorted $CD8^+$ TILS with 4-1BB treatment to lyse MMT tumor cells compared to those without 4-1BB treatment (Figure 8), again suggesting that 4-1BB agonistic antibody was effectively able to prevent the development of tolerance. This pilot study was performed in groups of three mice. At present a full study of six mice in each group (CTL, CTL + 4-1BB, 4-1BB alone, PBS) is underway.

Investigation of immune competence of breast cancer patients Since T cells and DCs are pivotal in the development of anti-tumor immunity and knowledge of their functionality is important in the development of the scheme for the clinical trial, we investigated DC and T cell function from twenty-three breast cancer patients at various stages of the disease and compared the data to eight normal age-matched donors. Although there have been several studies describing the functional impairment of T cells and DC in breast cancer patients, the studies have not evaluated both T cell and DC function from the same breast cancer patients. Moreover, the mechanisms driving the functional impairment still remain elusive. The goal of our study was to evaluate the immune status of patients presenting with the diagnosis of breast cancer and to evaluate the immune-modulating factors within the tumor milieu that may potentially account for the functional impairment of immune effector cells. This is the first study describing a thorough analysis of both T cell and DC function in newly diagnosed breast cancer patients ((10) see appendix). Impaired functionality of T cells and DCs correlated with COX-2 and PGE_2 over-expression. These studies are of critical importance for designing novel immunotherapeutic strategies for breast cancer but also in selecting the patients that may most benefit from such therapies.

Study Characteristics This research study was approved by the Mayo Clinic Institutional Review Board (IRB). Patients who presented to the Mayo Clinic Scottsdale Breast Clinic for initial treatment of disease were eligible for the protocol. The patients signed informed consents for peripheral blood and tumor samples. Informed consent for peripheral blood samples was also

obtained from healthy, age-matched volunteers. A total of twenty-three patients with breast cancer and eight healthy controls were studied. Breast cancer patient demographics and tumor characteristics are shown in Table 1. Nearly all patients were post-menopausal with a mean age of 69 years. Normal donors were all post-menopausal with a mean age of 60 years. Infiltrating ductal adenocarcinoma was the most common tumor subtype (57%). The mean size of the tumors was 2.4 cm, although 71% of lesions were 2 cm or less. Only 20% of patients had lymph node metastases and the majority of patients presented with either stage I or stage II disease. In most cases, blood was drawn on the day of surgery, prior to resection of the tumor. In some cases, blood was drawn few days prior to surgery.

The overall schematic for the study design is shown in Figure 9. Whole blood was obtained from the study subjects and the peripheral blood mononuclear cells (PBMC) were separated using a Ficoll-Paque density gradient centrifugation. Sera from these patients were also collected and stored in -80°C freezer. The PBMC were used for isolation of T cells and DCs and the serum was used for cytokine/chemokine and PGE_2 evaluation. Surgically resected tumor tissue and lymph node metastasis were used to make tissue lysate for COX-2 and PGE_2 evaluation.

T cells from patients with breast cancer show reduced proliferative abilities and reduced levels of immunostimulatory Th1 cytokines and increased levels of Th2 cytokines. The proliferative ability of T cells isolated from breast cancer patients just prior to surgical resection of tumor was examined. T cells were stimulated by various concentrations of plate-bound CD3 antibody and T cell proliferation measured by ^{3}H-thymidine uptake. T cell proliferation was significantly reduced in cancer patients compared to normal controls ($p<0.001$ at 1ug and 0.5ug/ml CD3 antibody) (Figure 10). Additional analysis of Th1/Th2 cytokines in activated cells revealed reduced intracellular levels of the immunostimulatory Th1 cytokines IFN-γ ($p<0.001$), TNF-α ($p<0.001$), IL-12 ($p=0.002$) and IL-2 ($p<0.001$), and increased levels of Th2 cytokines, IL-4 ($p=0.02$) and IL-10 ($p=0.003$), Figure 11.

DCs from breast cancer patients exhibit an immature phenotype. DCs from patients with breast cancer expressed reduced levels of co-stimulatory molecules such as CD80 (B7.1), CD86 (B7.2), and CD 40 upon maturation with LPS (Figure 12). Similar results were obtained with TNF-α-induced maturation. Other markers utilized to determine DC phenotype included HLA-DR, HLA-ABC, CD1a and CD14 (data not shown). No differences between cancer patient and normal donors were observed in these markers. The reductions in expression of both CD80 and CD86 were statistically significant, $p<0.001$. This low expression of co-stimulatory molecules is an indication that these DCs remain immature. It has been suggested that immature DCs possess reduced ability for stimulating T cells and therefore may contribute to tumor-induced T cell tolerance rather than immunity, and that mature DCs are essential for presenting tumor antigens and activating T cells to become cytolytic against tumor cells.

We therefore tested if DCs from these cancer patients had reduced antigen presentation. Data indicate that the function of DCs was significantly reduced. DCs from breast cancer patients demonstrated significantly reduced ability to present antigen to allogeneic normal T cells in a Mixed Lymphocyte Reaction (MLR), $p<0.001$ (Figure 13A). The MLR results support the previous findings shown in Figure 12 where the same DCs expressed low levels of co-stimulatory molecules and therefore failed to fully mature. Furthermore, the immature DCs from

breast cancer patients demonstrated significantly reduced ability to phagocytose exogenous antigens *in vitro*, $p<0.002$ (Figures 13B and C). They also demonstrated a maturation defect when stimulated with LPS treatment (Figures 13C). Figure 13B shows a representative histogram from one patient and Figure 13C shows a bar graph of average values from the 23 patients. Immature DCs are known to be strong phagocytes but weak antigen presenters whereas mature DCs are weak phagocytes and strong antigen presenters (11). We found that immature DCs from normal donors showed significantly higher ($p<0.002$) fluorescence intensity (mean intensity of 1620) demonstrating good phagocytic ability and upon maturation with LPS, their phagocytic activity was reduced (mean intensity of 265) (Figure 13C). In comparison, the immature DCs from the breast cancer patients had significantly lower fluorescence intensity (mean intensity of 270) indicating poor phagocytosis and the fluorescence intensity of these DCs did not decrease with LPS treatment (mean 220), once again indicating impaired maturation (Figure 13C). Similar results were obtained when TNF-α instead of LPS was used to mature DCs (data not shown).

Breast cancer patients show elevated levels of COX-2 and PGE2 levels in tumor milieu and in circulation. Tumor cells secrete factors that are known to induce immunosuppression and promote tumor cell proliferation. Prostaglandins, especially PGE_2, is one such factor expressed within the tumor microenvironment as well as secreted in the serum. Since COX-2 is the key regulator of prostaglandin synthesis, we evaluated the COX-2 protein expression on western blots of adjacent normal tissue lysates, tumor tissue lysates and lysates from lymph nodes that contained metastases. The COX-2 protein was over-expressed in both the tumor and lymph node metastases compared with normal tissue, with the highest expression being observed in lymph node metastases (Figure 14A). Next, we evaluated the amount of PGE_2 in the serum of breast cancer patients and normal donors by specific PGE_2 metabolite ELISA. Since PGE_2 is rapidly converted in the serum by 15-OH PGDH to its 13,14-dihydro-15-keto metabolite, direct measurement of intact PGE_2 is not possible in sera or plasma. Thus PGE_2 metabolite measurement is necessary to provide a reliable estimate of actual PGE_2 production (12, 13). However, in the tumor tissue lysates, PGE_2 levels can be evaluated directly using a specific ELISA for PGE_2 as these cell types do not contain the enzymes required for metabolism of PGE_2, thus keeping the PGE_2 levels stable. Breast cancer patients had significantly elevated levels of PGE_2 metabolite levels in their sera as compared to normal, $p<0.001$ (Figure 14C). Similar to COX-2 expression, we observed high levels of PGE_2 in the tumor cell lysates ($p=0.042$) and lymph node metastases ($p=0.017$) as compared to normal adjacent breast tissue (Figure 14B).

Since tumor lysates contained COX-2 and PGE_2 and presumably other immunosuppressive factors, we determined if these lysates could directly block T cell signal transduction and activation that lead to T cell proliferation. We evaluated the effect of the tumor lysates on the proliferation of T cells from the normal donors. We used purified PGE_2 as standard and compared it to the inhibition observed with the tumor lysate. There was direct inhibition of normal T cell proliferation in response to CD3 antibody by the tumor lysates and by purified PGE_2 (Figure 15). The inhibition observed with tumor lysate was higher than purified PGE_2, suggesting the presence of other T cell inhibitory agents in the tumor lysate.

We evaluated our data to see whether the patient's clinical variables correlated with immune function parameters. The clinical parameters examined were stage, lymph node status, estrogen receptor status, tumor size (≤2 cm vs. 2 cm), grade, presence of angiolymphatic invasion, multifocality, and history of previous breast cancer. No differences in T cell function or serum PGE_2 were noted among these groups. Dendritic cell immaturity and phagocytic ability were significantly higher ($p<0.05$) in patients with higher stage and patients with lymph node metastases. There were no significant differences among the other clinical parameters, which could be due to small sample size.

Methods

T Cell Isolation T cells were isolated from PBMC of patients as described in the schematic (Figure 1). Briefly, mononuclear cells were obtained by centrifugation of peripheral blood over Ficoll-Paque gradient (Amersham Biosciences, Sweden). Mononuclear cells were incubated for 2h at 37°C and non-adherent lymphocytes were removed and used as T cells and the adherent cells were used for generation of DCs.

T Cell Proliferation Assay Non-adherent lymphocyte population (1×10^6/ml) was subjected to *in vitro* stimulation with various concentrations of purified plate-bound CD3 antibody (BD Pharmingen, San Diego, CA). Cells were incubated for 4 days with CD3 antibody and ^{3}H-thymidine was added 24 hours prior to harvest. After washing off excess thymidine, cells were lysed with 5% Triton X-100, and incorporated thymidine was evaluated using the Topcount micro-scintillation counter (Packard Biosciences, Shelton, CT). Evaluation of T cell proliferation was also performed with varying concentrations of tumor lysates (12ug/ml-200ug/ml) and purified PGE_2 (Cayman Pharmaceutical, Ann Arbor, MI).

Analysis of Intracellular Cytokines Intracellular cytokine levels were evaluated by two-color flow cytometric analysis following TCR ligation. Intracellular cytokines were determined post brefeldin-A (BD Pharmingen) according to manufacturer's recommendation (4uls/1.2 X 10^7 cells/6mls for 3h at 37°C prior to staining). This treatment stops the release of cytokines in the culture media and the cytokines accumulate within the cells. Cells were then stained for surface markers for T cells (CD3) or DC (HLA-DR) at 4°C for 15 minutes followed by washing excess stain and permeabilizating cells with Pharmingen permeabilization solution (containing saponin) for 30 minutes at 4°C. Cells were then stained for intracellular IL-2, IL-12, IFN-γ, IL-4, IL-10, and TNF-α for 30 minutes at 4°C. Cells were analyzed using the Becton Dickinson FACScan and data analyzed using the Cell Quest Program. All antibodies were purchased from BD Pharmingen.

DC Isolation and Maturation DCs were generated from $CD14^+$ monocyte population isolated from PBMC. Briefly, mononuclear cells were obtained by centrifugation of peripheral blood over Ficoll-Paque gradient. Mononuclear cells were incubated for 2h at 37°C and non-adherent cells were removed. Adherent cells were incubated with GM-CSF (5ng/ml, Pepro Tech, Rocky Hill. NJ.) and IL-4 (5ng/ml, PeproTech) for 4 - 5 days. Cells were collected, counted, and phenotyped for immature DC and further cultured for one additional day with GM-CSF (5ng/ml), IL-4 (5ng/ml) and LPS (100ng/ml, Sigma Pharmaceuticals, St. Louis, MO). Cells were collected on day 6 as mature DC.

DC Phenotype Control and breast cancer patient DCs were analyzed by two-color flow cytometric analysis. Cell surface expression of several markers was evaluated: CD80 (B7-1), CD86 (B7-2), CD40, HLA-DR, HLA-ABC, CD1a and CD14. All antibodies were purchased from BD Pharmingen. Stained cells were analyzed utilizing the Cell Quest program on a Becton Dickinson FACScan.

DC Function A) Mixed Lymphocyte Reaction (MLR) Assay. Control and patient-derived DCs were assayed for their ability to stimulate allogeneic T cells in an MLR. T cells (1×10^5) from normal donors were incubated with irradiated DCs (3000 rads, 1×10^4 cells) from allogeneic breast cancer patients for a period of 5 days and ^{3}H-thymidine was added 24 hours prior to harvesting the cells. After washing off the excess thymidine, cells were lysed with 5% Triton X-100, and incorporated thymidine was evaluated using the Topcount micro-scintillation counter.

B) Phagocytosis ability. Immature and LPS-matured DCs from normal donors and breast cancer patients were incubated with FITC-conjugated dextran beads (MW: 40,000, Molecular Probes Inc., Eugene, OR) at 1 mg/1 x 10^6 cells for 30 minutes at 37° C. Dextran beads were used as the exogenous antigen source. Since the beads were conjugated to FITC, uptake of dextran beads by DCs was analyzed by flow cytometry and mean fluorescence intensity was calculated.

Breast Tumor Cell Lysates Tissue lysates were prepared within 1 hour post surgery by homogenizing the tumor tissue in lysate buffer containing 20 mM Hepes, 0.15M NaCL, and 1% Triton X-100 supplemented with phosphatase inhibitor cocktail mix (1:100 dilution, Sigma Pharmaceuticals) and complete protease inhibitors (Roche Pharmaceuticals, Indianapolis, IN).

Expression of COX-2 Protein in Tumor Lysate Protein concentrations of the lysates were determined by Pierce BCA protein assay kit (Pierce, Rockford, IL). SDS-PAGE electrophoresis was performed using 12% resolving gel. 100 µgs of protein was loaded per lane. Gels were immunoblotted and probed for COX-2 with specific COX-2 monoclonal antibody (goat polyclonal, clone C20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:200 dilution.

PGE_2 in Serum and Tumor Lysate Levels in the lysates were determined using a specific ELISA kit for PGE_2 and levels in the serum were determined using the PGE_2 metabolite ELISA kit (Cayman Pharmaceuticals, Ann Arbor, MI). Manufacturer's recommended protocols were followed.

Statistical Analysis Statistical significance was assessed using pair-wise comparisons with the Tukey-Kramer adjustment for multiple comparisons. The margins-of-error for the comparisons were obtained by calculating the 95% confidence intervals for the differences between group proportions.

Immune function parameters were compared between a set of clinical indicators. The clinical parameters examined were stage, lymph node status, estrogen receptor status, tumor size (<2 cm vs. 2 cm), grade, presence of angiolymphatic invasion, multifocality, and history of previous breast cancer. Due to the non-normality of the immune function data and the small sample size of the cohort, the exact Wilcoxon statistic was used in assessing significant differences between the groups. All error bars in the figures represent the standard deviation of the mean.

Specific Aim 2: To translate the most effective vaccine strategies into phase I clinical trials in patients with high and low tumor burden.

We are including the draft of the clinical trial that is presently being submitted to the Mayo Comprehensive Cancer Center Concept Committee prior to submission to the Institutional Review Board (IRB) and to the Federal Drug Administration (FDA) for an IND, which will be pursued concomitantly. Although we originally proposed to use patients with high and low tumor burden, our findings regarding patients with high tumor burden suggested that this category of patient was not optimal for immunotherapy. Patients with breast cancer have T cells that showed decreased proliferation in response to T cell receptor stimulation, dendritic cells with reduced levels of co-stimulatory molecules and reduced ability to mature in response to stimulation, as well as increased PGE_2 (an immunosuppressive protein) levels in circulation. These factors suggest that individuals with tumor burden would not be optimally responsive to immunotherapeutic strategies. Thus, we have chosen to test our cancer vaccine in patients free of detectable breast cancer at the time of registration. Patients will have had histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy and have completed "standard therapy" three months prior to enrollment in our clinical trial. Patients must have MUC1-positive breast cancer (90% of breast cancers are MUC1 positive) and be HER-2 positive as defined by HercepTest (1+, 2+, 3+). Patients must be HLA-A2 positive. We will enroll 40 patients total at Mayo Clinic Rochester, Mayo Clinic Scottsdale, and Mayo Clinic Jacksonville. We can easily meet this criterion of patient number. The trial will test MUC1 class I peptide, HER-2/neu class I peptide and HER-2/neu class II peptide (two doses of each peptide) delivered in Montanide ISA-51 and compare GM-CSF with unmethylated CpG oligodeoxynucleotides as immune adjuvants. Few vaccines have been tested in the optimal setting of minimal residual disease. We feel this is a strength of this trial. The clinical trial entitled "MUC1/HER-2neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas" is included in the appendix. It is still in draft form as it is being submitted to the Mayo Comprehensive Cancer Center Concept Committee prior to IRB submission. The decision on the precise dosing of the CpG oligodeoxynucleotide is still under consideration and the precise pharmacy information for CpG and the peptides will be added once the CpG dose is established and the peptides have been manufactured by Clinalfa.

KEY RESEARCH ACCOMPLISHMENTS

- Two additional immunization strategies have been tested in our mouse model of spontaneous mammary gland cancer (MMT mice).
    - Dendritic cells pulsed with tumor lysate
    - Adoptive transfer of MUC1-specific cytotoxic T lymphocytes
- Tumor microenvironment induced tolerance as shown by failure of T cells to proliferate to MUC1 and reduced lytic capabilities.
- Cross-linking of co-stimulatory molecules strategies to reverse established tolerance were tested.

  - Dendritic cells pulsed with tumor lysate plus OX40
  - Cytotoxic T lymphocytes with agonistic antibody to 4-1BB

- Dendritic cells pulsed with tumor lysate plus OX40 failed to substantially overcome tolerance in MMT mice

- 4-1BB agonistic antibody plus MUC1-specific CTLs were effective in:

  - Greatly reducing tumor burden in immunized MMT mice
  - Overcoming T cell anergy/tolerance

- Investigation of T cell and DC functionality in breast cancer patients at time of diagnosis compared to age-matched volunteers showed reduced functionality.

  - T cell proliferation in response to T cell receptor antibody was significantly reduced in cancer patients compared to normal controls ($p<0.001$).

  - Cancer patients exhibited significantly reduced intracellular levels of Th1 cytokines and significantly increased levels of Th2 cytokines compared to normal controls.

  - Dendritic cells from breast cancer patients expressed significantly reduced levels of co-stimulatory molecules (CD80, CD86, CD40) upon maturation with LPS or TNFα.

  - Dendritic cells from breast cancer patients had significantly reduced ability to present antigens to allogeneic normal T cells ($p<0.001$).

  - Dendritic cells from breast cancer patients had significantly reduced ability to phagocytose antigens ($p<0.002$).

- COX-2 protein was over expressed in breast tumor tissue and lymph node metastases compared to normal breast tissue.

- Breast cancer patients had significantly elevated levels of PGE_2 metabolite levels in sera. PGE_2 is known to induce immunosuppression. PGE_2 was also elevated in tumor cell lysates and lymph node metastases compared to normal adjacent breast tissue.

- A draft of the Clinical Trial is included in the appendix.

REPORTABLE OUTCOMES

Mukherjee, P., Madsen, C.S., Ginardi, A.R., Tinder, T.L., Jacobs, F., Parker, J., Agrawal, B., Longenecker, B.M. and Gendler, S.J. (2003) MUC1-specific immunotherapy in a mouse model of spontaneous breast cancer. J. Immunother 26:47-62.

Xia J, Tanaka Y, Koido S, Liu C, Mukherjee P, Gendler SJ and Gong J (2003) Prevention of spontaneous breast carcinoma by prophylactic vaccination with dendritic/tumor fusion cells. J Immunol 170:1980-1986.

Pockaj, B.A., Basu, G.D., Pathangey, L.B., Gray, R.J., Hernandez, J.L., Gendler, S.J. and Mukherjee, P. (2003) Reduced T cell and dendritic cell function is related to COX-2 over-expression and PGE2 secretion in patients with breast cancer. Annals of Surgical Oncology, under revision.

Mukherjee, P., Basu, G., Pockaj, B., Pathangey., Ginardi, A.R., and Gendler, S.J. Reduced T cell and dendritic cell function in patients with breast cancer. Days of Molecular Medicine: Immunotherapy (A Technology Platform for Molecular Medicine). March 2003.

Mukherjee, P., Basu, G.D., Pathangey, L.B., Pockaj, B.A., Tinder, T.L., Ginardi, A.R. and Gendler, S.J. COX-2 induced PGE2 synthesis is involved in downregulation of immune cell function in patients with breast cancer, American Association of Immunologists, May 2003.

Basu, G., LaGioia, M., Tinder, T., Bradley, J., Pathangey, L., Gendler, S.J., and Mukherjee, P. The cyclo-oxygenase 2-selective inhibitor, celecoxib, mediates growth inhibition in breast cancer cell lines via diverse pathways. AACR, Frontiers in Cancer Prevention Research, July, 2003.

Pinku Mukherjee, Ph.D. was awarded a 3-year grant from The Susan G. Komen Breast Cancer Foundation (BCTR0202089) for a total of $250,000. The grant is entitled "Anti-tumor synergy between targeted COX-2 inhibition, low-dose chemotherapy and immune-based therapies for treatment of metastatic breast cancer". Sandra Gendler is a co-investigator.

Pinku Mukherjee, Ph.D. received the 2003 Junior Faculty Award at the American Association of Immunologists 90th Annual Meeting in May, 2003.

CONCLUSIONS

All of the vaccine strategies have elicited an immune response. Animals developed mature CTLs which were lytic *in vitro* against MUC1-expressing tumor cells. Lytic activity was detected without further *in vitro* stimulation. However, in most cases the spontaneous tumors progressed. The CTLs, while active outside of the environment of the tumor, were tolerized and unreactive to MUC1 (a target antigen) in the vicinity of the tumor. Tumors were found to exhibit several known escape mechanisms, such as the production of immunosuppressive factors and down-modulation of MHC class I molecules on the cells. CTLs adoptively transferred into the tumor-bearing mice were used to follow the development of tolerance, which occurred within about three weeks following injection. Stimulation of co-stimulatory molecules, especially 4-1BB, a member of the TNFR family, together with adoptive transfer of MUC1-specific T cells, resulted in substantially reduced tumor burden in MMT mice. The CTLs appeared to remain lytic against the tumor in the tumor environment and tolerance/anergy was alleviated. CTLs recovered from the tumors were lytic against MUC1-expressing tumor cells (MMT tumor cells in culture, as well as B16/MUC1 cells) and they proliferated in response to DCs presenting MUC1. Although 4-

1BB is not yet ready for clinical trials, co-stimulation is definitely one direction to pursue in future trials.

In preparation for the clinical trial we are about to embark upon (Aim 2 of this proposal), we investigated the T cell and dendritic cell functionality in patients with breast cancer prior to surgery. Patients studied were mainly stage 1 and 2 breast cancers. In a number of cancers, the immune system is not fully functional and immunosuppressive factors have been reported to be present. Ours is the first study to evaluate dendritic cell (DC) and T cell function from the same breast cancer patients and from age-matched controls. We found reduced functionality in both T cells and DCs in breast cancer patients. T cell proliferation in response to T cell receptor stimulation was significantly reduced, and T cells exhibited reduced intracellular levels of immunostimulatory Th1 cytokines and increased intracellular levels of Th2 cytokines compared to normal controls. Dendritic cells appeared to be of the immature phenotype, even following maturation with LPS or TNFα. Significantly reduced levels of co-stimulatory molecules (CD80, CD86, CD40) were expressed following maturation, there was reduced ability to present antigens to allogeneic normal T cells and significantly reduced ability to phagocytize antigens. All of these factors suggest that patients with tumor burden, whether high or low, would not be optimal candidates for immunotherapeutic strategies. In addition, we found high levels of COX-2 and prostaglandin E_2, proteins that could contribute toward immunosuppression. If the immune system of breast cancer patients were persistently compromised, the success of immunotherapeutic strategies for cancer will be limited unless the immune system can be appropriately stimulated. This information caused us to design our clinical trial for breast cancer patients with no evidence of disease, with the vaccine to be given three months following surgery and adjuvant chemotherapy and/or radiation therapy. This time lapse should allow the immune system to recover to normal levels prior to the first immunization. T cell and DC functionality will be determined prior to immunization. The draft clinical trial is included in the appendix. It is presently being considered in the Mayo Comprehensive Cancer Center Concept Committee prior to IRB submission and FDA submission to obtain an IND.

Future Studies

We are including the draft of the clinical trial that is presently being submitted to the Mayo Comprehensive Cancer Center Concept Committee prior to submission to the Institutional Review Board (IRB) and to the Federal Drug Administration (FDA) for an IND, which will be pursued concomitantly. We will enroll patients free of detectable breast cancer at the time of registration. Patients will have had histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy and have completed "standard therapy" three months prior to enrollment in our clinical trial. Patients must have MUC1-positive breast cancer (90% of breast cancers are MUC1 positive) and be HER-2 positive as defined by HercepTest (1+, 2+, 3+). Patients must be HLA-A2 positive. We will enroll 40 patients total at Mayo Clinic Rochester, Mayo Clinic Scottsdale, and Mayo Clinic Jacksonville. We can easily meet this criterion of patient number. The trial will test MUC1 class I peptide, HER-2/neu class I peptide and HER-2/neu class II peptide (two doses of each peptide) delivered in Montanide ISA-51 and compare GM-CSF with unmethylated CpG oligodeoxynucleotides as immune adjuvants. Few vaccines have been tested in the optimal setting of minimal residual disease. We feel this is a strength of this trial. The draft of the clinical trial entitled "MUC1/HER-2neu Peptide Based

Immunotherapeutic Vaccines for Breast Adenocarcinomas" is included in the appendix. The decision on the precise dosing of the CpG oligodeoxynucleotide is still under consideration and the precise pharmacy information for CpG and the peptides will be added once the CpG dose is established and the peptides have been manufactured by Clinalfa.

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APPENDICES

Figures 1 thru 15

Table 1

Draft Clinical Trial

Mukherjee, P., Madsen, C.S., Ginardi, A.R., Tinder, T.L., Jacobs, F., Parker, J., Agrawal, B., Longenecker, B.M. and **Gendler, S.J.** (2003) MUC1-specific immunotherapy in a mouse model of spontaneous breast cancer. J Immunother 26:47-62.

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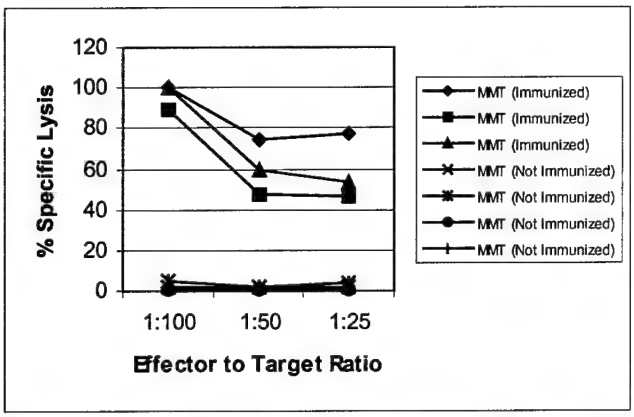


Figure 1. Immunization elicits mature MUC1-specific CTLs in MMT mice. Mature MUC1-specific CTL activity was detected by a standard 8 hr. ^{51}Cr-release assay using B16 melanoma cells transfected with human MUC1 as targets and draining lymph node T cells from MMT mice as effectors.

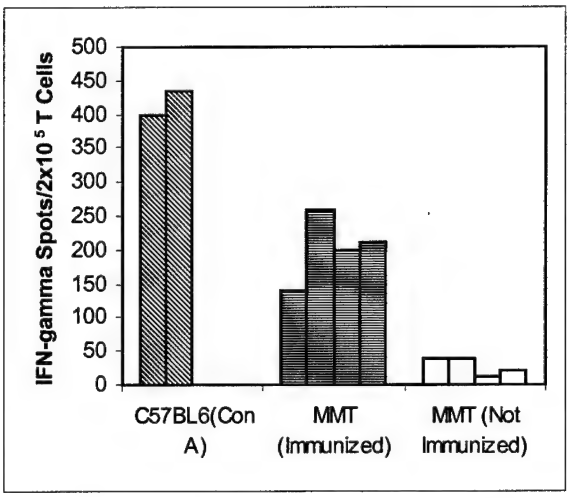


Figure 2. Elispot assay showing increased IFN-γ production by T cells in immunized MMT mice. Splenic T cells from MMT mice were isolated and stimulated with DCs pulsed with MUC1 peptide for 3 days. The stimulated T cells were then seeded at $2x10^5$ cells per well in Elispot plates and resulting spots were evaluated after 48hrs. For positive controls T cells from wildtype C57BL/6 mice were stimulated with Con A.

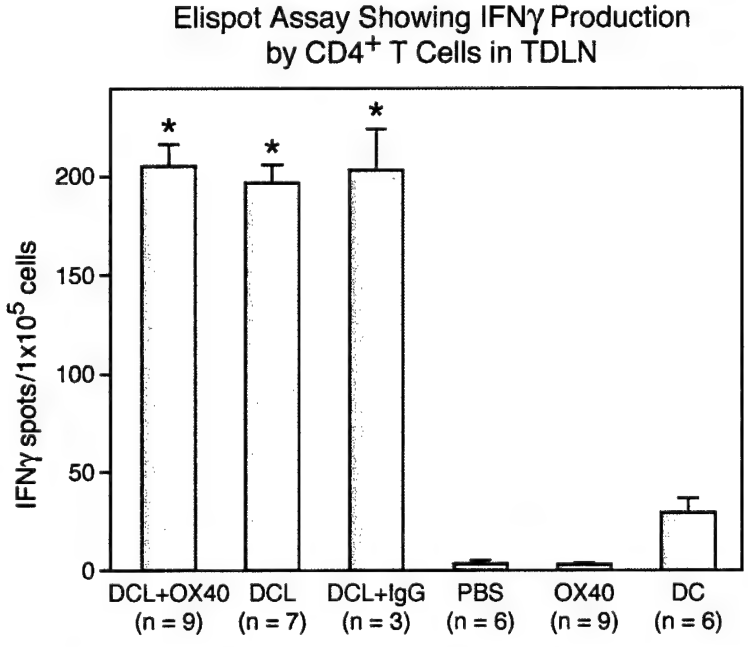


Figure 3. Splenic T cells from MMT mice were isolated and stimulated with DCs pulsed with MUC1 peptide for 3 days. The stimulated T cells were then seeded at $1x10^5$ cells per well in Elispot plates and resulting spots were evaluated after 48hrs.

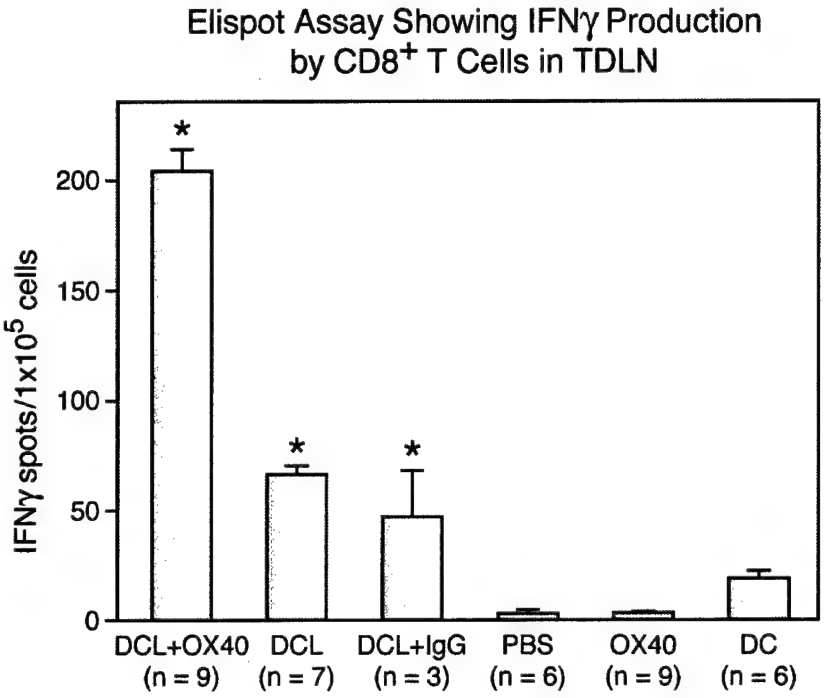


Figure 4. Splenic T cells from MMT mice were isolated and stimulated with DCs pulsed with MUC1 peptide for 3 days. The stimulated T cells were then seeded at $1x10^5$ cells per well in Elispot plates and resulting spots were evaluated after 48hrs.

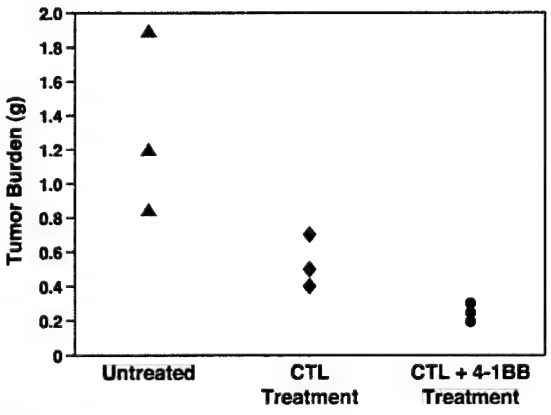


Figure 5. Tumor burden was greatly reduced in CTL treated and CTL + 4-1BB treated MMT mice. CTLs (2×10^7 iv) were given at weeks 9, 12, and 15; 4-1BB agonistic antibody (100 ug/mouse) was given weekly from weeks 10 to 16. Mice were sacrificed at 17 weeks. Tumor burden was determined by palpation and calculated by the formula: gm = (length x width^2) x 0.5 where length and width were measured in cm.

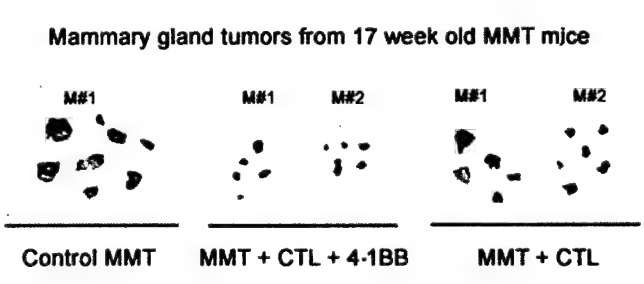


Figure 6. Photographic image of tumors dissected from mammary glands of treated and control mice.

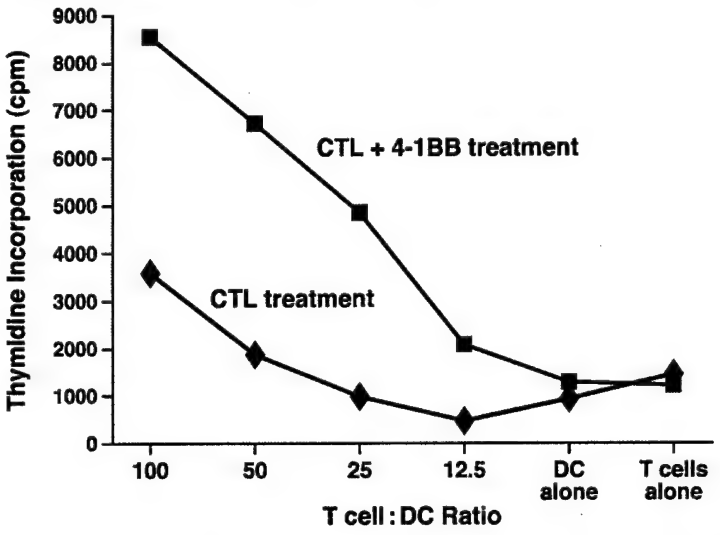


Figure 7. Ability of T cells to proliferate in response to MUC1 presented on irradiated DC was enhanced with 4-1BB treatment. T cells ($CD8^+/V\beta5^+$) sorted from the tumors were cultured with DCs pulsed with MMT tumor lysate (20 ug/ml) for 5 days and proliferation was measured by ^{3}H-thymidine incorporation.

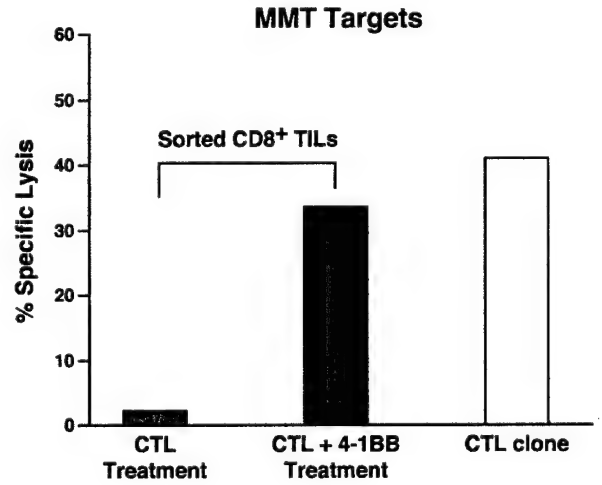


Figure 8. CTLs adoptively transferred with 4-1BB treatment remained lytic in tumor environment. Mice were sacrificed at 17 weeks. CTL activity of T cells ($CD8^+/V\beta5^+$) sorted from tumors was determined by standard ^{51}Cr-release assay using MMT cells as target cells. Effector cells were not stimulated *in vitro* prior to the assay. In untreated mice, no CTL activity has ever been detected. Assay was performed at effector to target ratio of 100:1. MUC1-specific CTL clone maintained in culture was used as positive control.

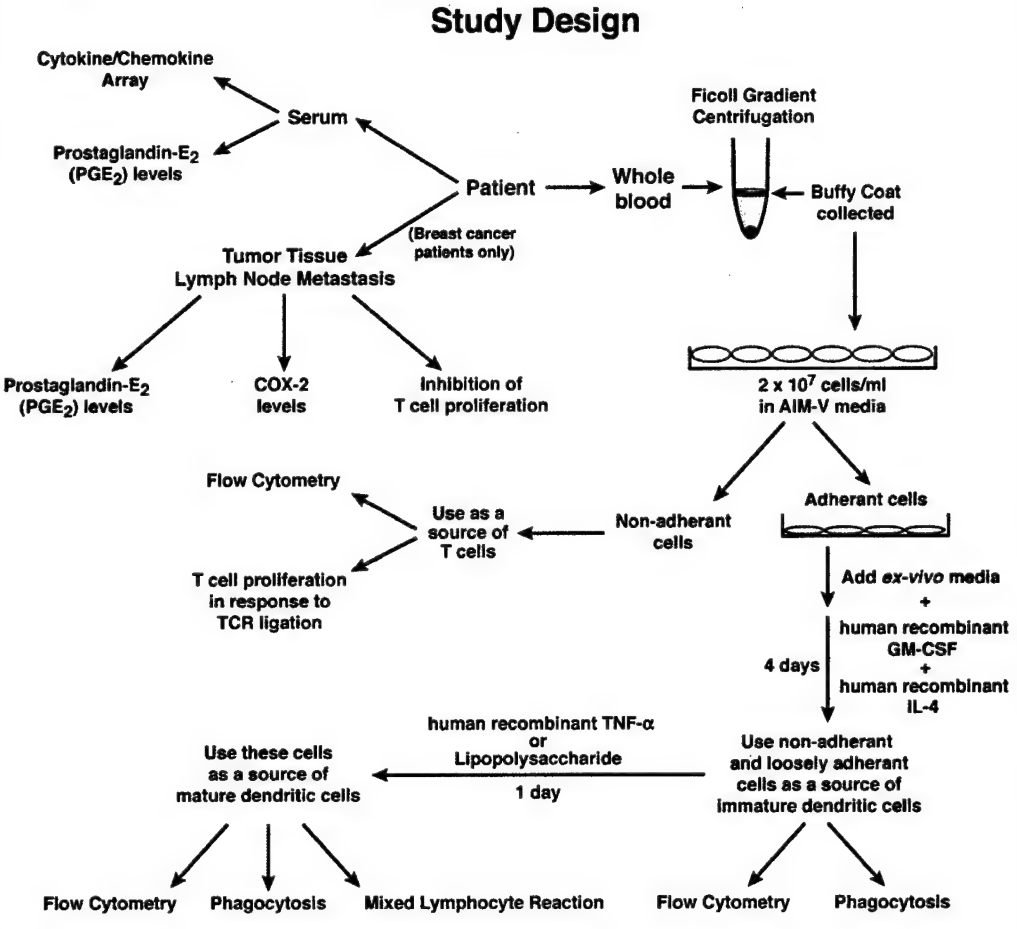


Figure 9. Schematic representation of the study design.

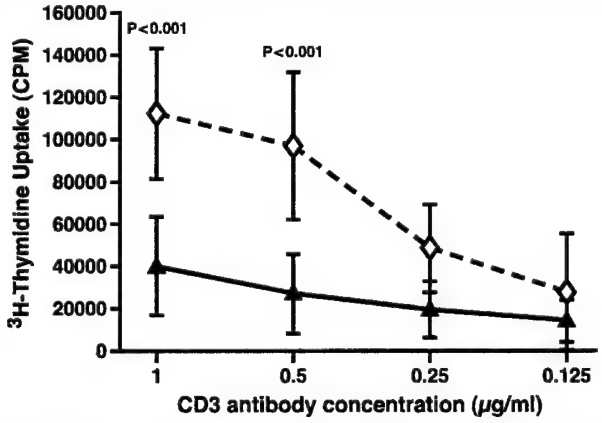


Figure 10. T cells from breast cancer patients have markedly reduced proliferation in response to specific TCR ligation. T cell proliferation in response to plate-bound CD3 antibody comparing breast cancer patients (____ , n = 23) and normal controls (_ _ _ _ , n = 8). Amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. Error bars represent standard deviation of the mean.

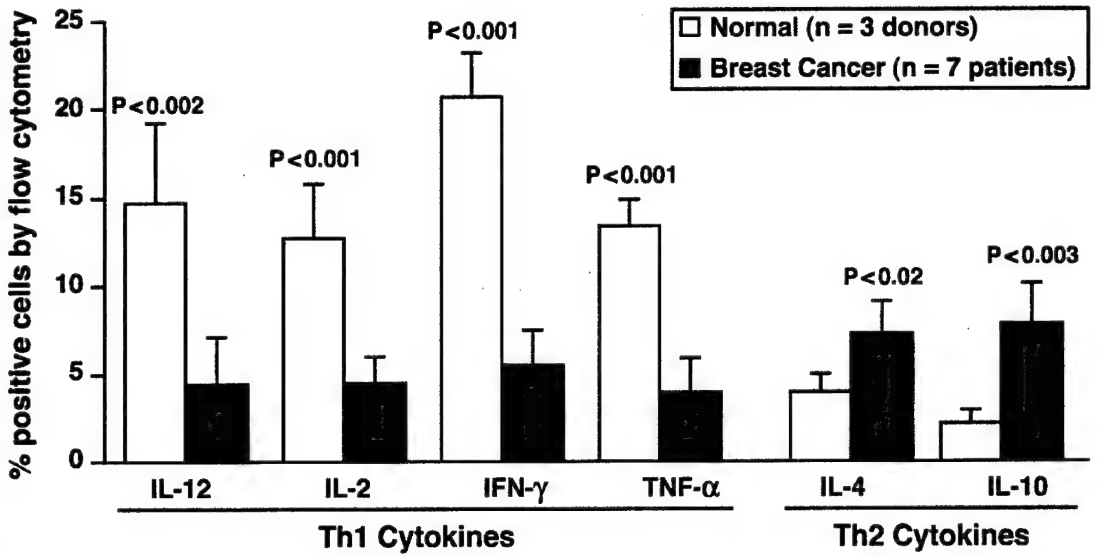


Figure 11. Reduced Th1-type cytokine and increased Th2-type cytokine in peripheral blood of patients with breast cancer. Comparison intracellular cytokine levels by flow cytometry. IFN-γ, TNF-α, IL-12, IL-2, IL-10 and IL-4 levels were compared between breast cancer patients (solid bars, n = 7) and normal controls (open bars, n = 3). Error bars represent standard deviation of the mean.

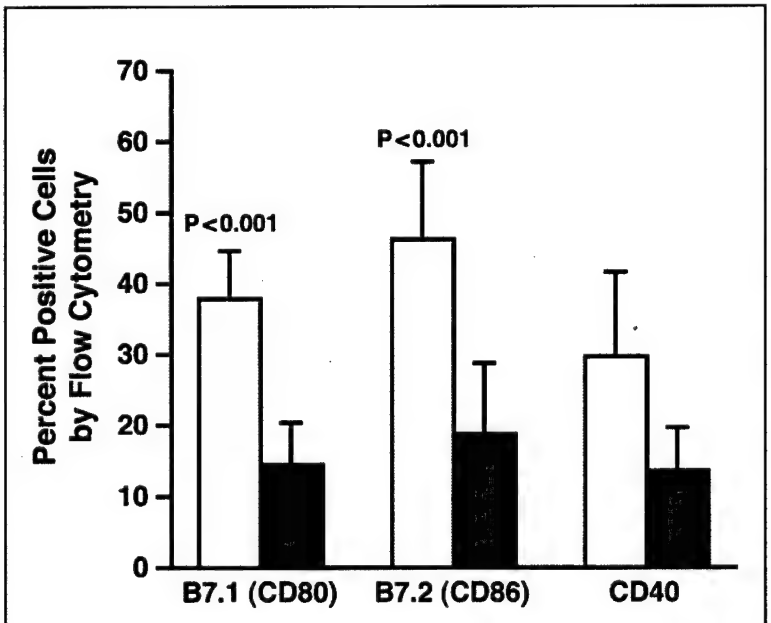


Figure 12. DCs from patients with breast cancer expressed reduced levels of co-stimulatory molecules. Flow cytometric analysis of surface expression of co-stimulatory molecules, CD80 (B7.1), CD86 (B7.2), and CD40 on LPS-matured DCs of breast cancer patients (solid bars, n = 23) vs. normal controls (open bars, n = 8). Similar results were obtained with TNF-α-matured DCs (data not shown). Error bars represent standard deviation of the mean.

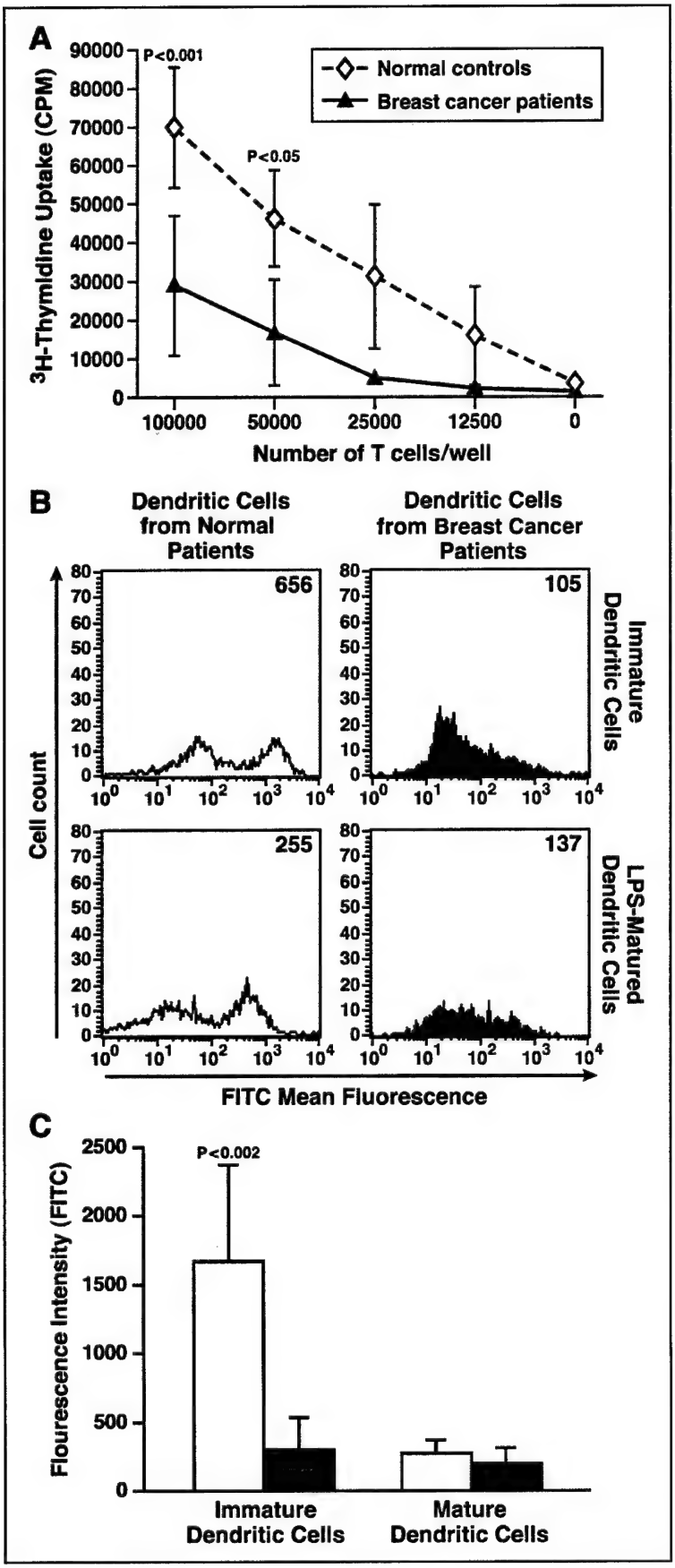


Figure 13. DCs from breast cancer patients demonstrate significantly reduced ability to present antigen to allogeneic normal T cells and demonstrates reduced phagocytosis of exogenous antigen. A) Allogeneic antigen presentation to normal T cells by DCs of breast cancer patients (◊ n = 23) vs. normal controls (▲ n = 8) in a Mixed Lymphocyte Reaction. Amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. B) Representative histogram of immature and LPS-matured dendritic cell phagocytic ability of a cancer patient versus normal donor (the numbers on the right corner are the mean fluorescence intensity) C) Dendritic cell phagocytic ability (mean fluorescence intensity) in breast cancer patients (solid bars, n = 23) vs. normal controls (open bars, n = 8) in mature and immature state. In 5B and C, mean fluorescence intensity is used as a measure for the amount of FITC-conjugated dextran beads engulfed by the DCs. Error bars represent standard deviation of the mean.

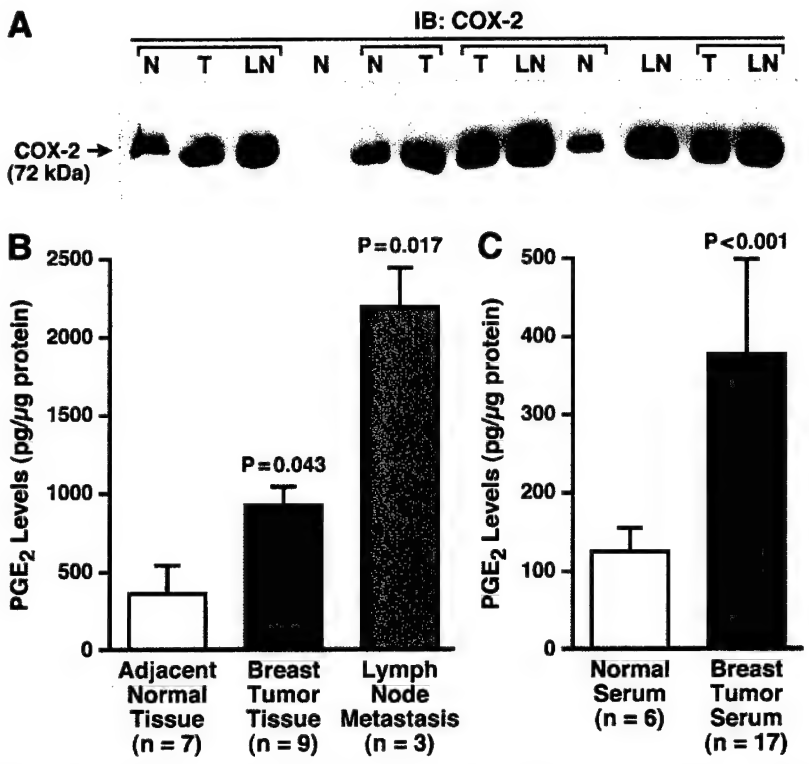


Figure 14. COX-2 and PGE2 is over-expressed in tumor tissue and serum of patients with breast cancer. A) COX-2 protein levels in tissue lysates (100ug) from adjacent normal breast tissue, breast tumors, and lymph node metastases were analyzed by western blot analysis. Brackets indicate tissues from a single patient. Six patient samples were analyzed. B) PGE_2 levels in tissue lysates from adjacent normal breast tissue, breast tumors, and lymph node metastases were determined by specific ELISA. C) Serum PGE_2 metabolite levels in breast cancer patients (solid bars) vs. normal controls (open bars) were analyzed by specific PGE_2 metabolite ELISA. Error bars represent standard deviation of the mean.

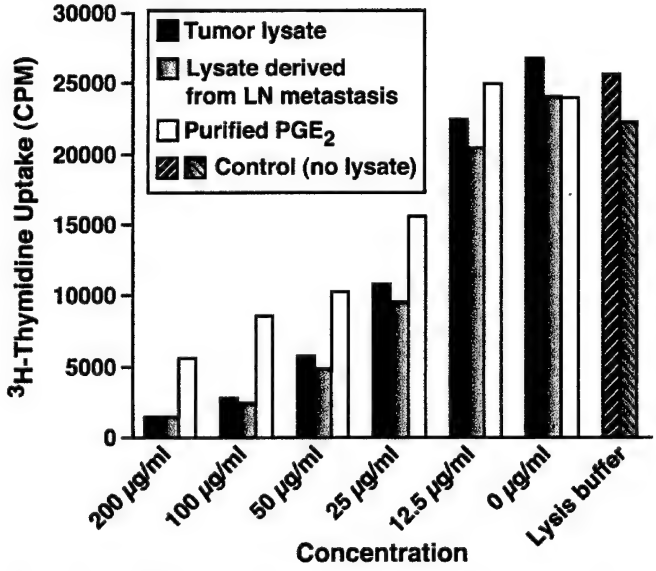


Figure 15. Direct inhibition of normal T cell proliferation by factors present in the tumor lysate. T cell proliferation was determined in response to plate-bound CD3 antibody in the presence or absence of tumor lysate or purified PGE_2 at varying concentrations (0ug/ml – 200ug/ml). T cells were generated from normal donors (n = 4). Amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. Tissue lysis buffer was used as the negative control and purified PGE_2 was used as the positive control.

Table 1. Patient and Tumor Characteristics

Mean Age (Range)	69 years (36-80 years)
Pre-Menopausal	1 (4%)
History of Contralateral Breast Cancer	4 (17%)
Tumor Type	
DCIS	1 (4%)
Infiltrating Ductal	13 (57%)
Infiltrating Lobular	4 (17%)
Mixed Infiltrating Ductal/Lobular	2 (9%)
Infiltrating Mucinous	3 (13%)
Mean Tumor Size (Range)	2.4 cm (0.3-13 cm)
Tumor $\leq$ 2 cm	15 (71%)
Bloom Richard Grade	
1	8 (38%)
2	5 (24%)
3	8 (38%)
Estrogen Receptor Positive	19 (83%)
Lymph Node Metastases	5 (24%)
Number of Lymph Nodes Positive	1-8
Stage	
0	1 (4%)
1	10 (43%)
2	10 (43%)
3	1 (4%)
4	1 (4%)
5	1 (4%)

Mayo Clinic Cancer Center

MUC1/HER-2/neu Peptide Based Immunotherapeutic Vaccines for Breast Adenocarcinomas

Principal Investigators/Study Chairs:

Svetomir Markovic, M.D., Ph.D. *
Sandra J. Gendler, Ph.D.#
Mayo Clinic Cancer Center
200 First Street, SW
Rochester, MN 55905
507/284–2511
507/284-5280 (FAX)

Mayo Clinic Scottsdale
13400 E. Shea Boulevard
Scottsdale, AZ 85255
480/301-7062
480/301-7017 (FAX)

Study Co-chairs:

Esteban Celis, M.D., Ph.D.*
James N. Ingle, M.D.*
Pinku Mukherjee, Ph.D.#
Peter J. Wettstein, Ph.D.*
Tom Fitch, M.D.#
Barbara Pockaj, M.D.#

Statistician:

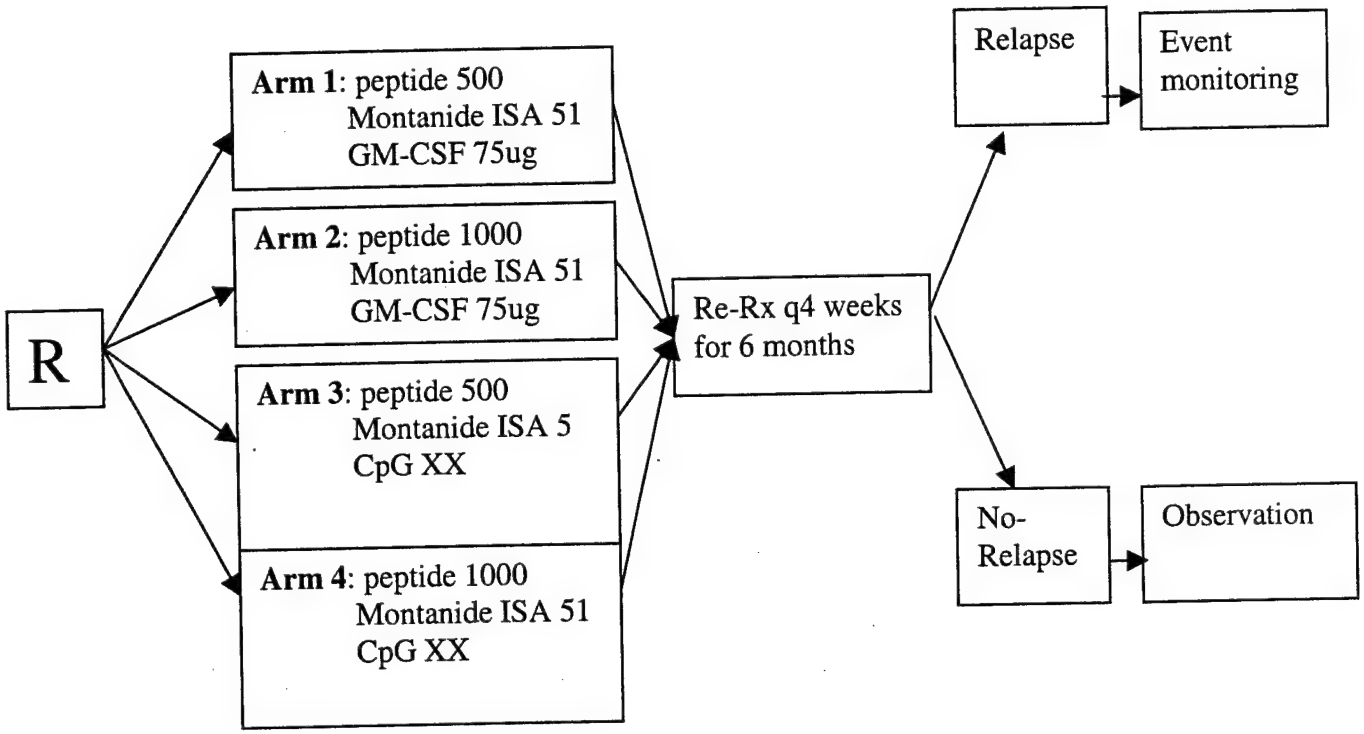
Vera J. Suman, Ph.D.

* Investigator having primary responsibility for this protocol
# Study Co-chair not responsible for patient care

Index

Schema

Prior to checking eligibility, contact the Randomization Center (507/284–2753) for study status and dose level.



1.0 Background

1.1 Breast cancer is diagnosed in 200,000 individuals in the United States annually and contributes to approximately 40,000 deaths each year. For tumors confined to the breast, surgical removal provides a good prognosis. However, primary tumor that metastasizes to distant sites, such as lymph nodes, lungs, liver and brain, correlates with a poor prognosis. Patients with advanced stage breast cancer are at high risk of relapse. Complications from metastatic disease are the leading causes of cancer-related deaths. Novel adjuvant strategies, such as breast cancer specific vaccines, are being considered as a clinical intervention that may reduce the chance of recurrence.

1.2 In recent years there has been great interest in the development of these cancer vaccines, which are designed to immunize individuals to antigens present on tumors. Cancer vaccines are a non-toxic therapy, which have been shown in several melanoma trials to be as effective as chemotherapy without the toxic side effects (1, 2). Because tumors can be surgically removed and there is often a long period of time before the tumor recurs at metastatic sites, cancer vaccines have been proposed as an optimal therapy that could prolong the time to recurrence. This optimal opportunity of immunization in the situation of minimal residual disease has rarely been tested, however, as most vaccines have been given to patients with large tumor burden after the failure of standard therapies in Phase I and Phase II trials.

1.3 The past two decades in tumor immunology have lead to the discovery of specific tumor antigens that have been shown in preclinical studies to elicit tumor-specific immunity and establish long term memory without autoimmunity. For breast cancer, vaccines composed of epitopes derived of MUC1, HER-2/neu, MAGE3, CEA have been studied and shown to be immunogenic without causing autoimmunity (3-6).

1.4 It is now clear that tumor antigens are presented in the context of specific class I and Class II HLA molecules. Class I presentation, in the presence of appropriate co-stimulation, is thought to stimulate a cytolytic $CD8^+$ T cell response, while antigen presentation in the context of Class II molecules stimulates a $CD4^+$ helper T cell response (7).

1.5 One approach for the development of a cancer vaccine is the use of tumor associated synthetic antigens for immunologic priming. Because specific peptides are ubiquitous in tumors of the same histologic type, identical peptide vaccines may be employed in allogeneic hosts bearing the same tumor histology. Additionally, the use of single peptides for immunization limits the potential induction of undesired autoimmunity (8-10). Recent developments in the use of soluble MHC Class I/peptide tetramers and elispot technology have enabled rapid characterization of epitope-specific CTL responses (11, 12). In addition to being well-explored and understood, many of these antigens are shared tumor antigens.

Vaccines that are composed of these antigens can be developed for use in a large number of patients. The primary limitations to peptide based vaccine strategies are haplotype restriction, potential for degradation, and uncertainty regarding which peptides, used alone or in combination, are the most immunogenic (13, 14). This study is designed to test these uncertainties.

1.6 One attractive and broadly applicable target for immunotherapeutic strategies is the MUC1 tumor antigen. MUC1, a cell-associated mucin, is expressed on the cell surface of many epithelial malignancies as well as by hematological malignancies (15-18). These include multiple myeloma (92%) and acute myelogenous leukemia (67%) (19). Greater than 90% of breast carcinomas express MUC1; high levels are also found in adenocarcinomas originating from most tissues (15, 17). MUC1 expression is greatly up-regulated on tumors (reviewed in Gendler (20)). Expression on tumors is no longer apical, but it is found all around the cell surface and in the cytoplasm. In addition, glycosylation on tumor-synthesized MUC1 is aberrant, with greater exposure of the peptide core than is found in normal tissues. MUC1 has long been an interesting target molecule for immunotherapeutic strategies, given its high level and ubiquitous expression. Patients with tumors, especially with breast, pancreas and ovarian tumors, have exhibited immune responses to MUC1 with the presence of antibodies and T cells specific for MUC1 detected in about 10% of individuals. An HLA unrestricted T cell response among cancer patients has also been described (21-24). There is increasing evidence from murine and human studies that MHC-restricted T cells can be induced in mice and humans after immunization with the MUC1 peptide or MUC1 antigenic epitopes (25-33). Importantly, there have been reports of two HLA-A2 binding peptides derived from the MUC1 protein (34). One of the peptides is from the tandem repeat sequence of MUC1 and the second peptide is from the signal sequence. MUC1-specific cytotoxic T cells (CTLs) have been induced in T cells from healthy donors following *in vitro* immunization using peptide-pulsed dendritic cells. MUC1-specific CTLs have also been induced *in vivo* after vaccination of breast and ovarian cancer patients with peptide-pulsed DCs (19).

1.7 A second candidate for peptide-based immunotherapy is HER-2/neu, the gene product of the erbB2/neu protooncogene. HER-2/neu is overexpressed in approximately 30% of breast cancer patients. HER-2/neu is also expressed by multiple types of tumors, including ovarian, lung, colon, pancreas and gastric tumors (35-37). HER-2/neu has particular relevance, as it is expressed at high levels in early *in situ* lesions in breast carcinoma (38). Thus, it is a target for early disease. Immunologic responses to HER-2/neu have been detected in a minority of patients with advanced stage breast and ovarian cancer, including antibodies, T helper and CD8 responses (39, 40). Several HLA-class I binding peptides have been previously identified. We recently identified a novel HLA-A2.1 binding peptide from the HER-2/neu extracellular domain [HER-2(9_{435})] (41). This peptide (ILHNGAYSL) bound to HLA-A2.1 with intermediate affinity (IC50 74.6 nM). The HER-2(9_{435}) epitope was tested using an *in vitro* immunization protocol and found to elicit CTLs that killed peptide-sensitized target cells. The

CTLs elicited also recognized the HER-2/neu antigens, as it specifically killed tumor cells expressing the HLA-A2.1 and HER-2/neu antigens (see below in preliminary data). Furthermore, recognition of the tumor cell targets was significantly inhibited by unlabeled (cold) targets pulsed with HER-2(9_{435}), but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (see below). Thus, the CTLs induced by HER-2(9_{435}) are antigen specific.

A potential limiting factor for peptide based immunotherapy is related to a defined antigenic repertoire which is HLA restricted. This factor, inherent to all peptide-based approaches, restricts patient access. Additionally, because individual peptides only have the potential to induce epitope-specific CTL, the vast majority of potential tumor antigens are not targeted. In this setting, tumor down regulation of individual antigens or HLA epitopes promotes immune evasion. Recent evidence, however, suggests that this problem of epitope restriction may not be as physiologically important as was previously postulated. Specifically, it has now been clearly demonstrated that a T cell response induced against one epitope can stimulate CTL response to other target epitopes through a mechanism termed epitope spreading (4, 42, 43). Using an experimental autoimmune encephalitis model, Vanderlugt et al. have demonstrated that disease progression is associated with the development of epitope-specific helper T cells, which are distinct from those initiating the disease. Transfer of secondary $CD4^+$ cells to naïve mice induces the disease phenotype and the disease is abrogated by blocking the secondary T cell response even though the primary T cell response remains intact (44, 45). Disis demonstrated epitope spreading in 84% of patients vaccinated with HER-2/neu peptides, reflecting the initiation of an endogenous immune response. The immunity persisted after active immunizations ended (4). These data suggest that peptide based approaches to cancer immunotherapy may indirectly stimulate multiple tumor reactive CTL against minor antigens in the presence of residual tumor. Based on this concept, the current study is designed as a therapeutic approach, with peptide epitope selection designed to enhance the number of potential candidates.

In addition to class I epitopes, immunogenic HLA-DR restricted class II epitopes have been defined for HER-2/neu. $CD4^+$ helper T lymphocytes (T_H) responses play an essential role in immunologically mediated anti-tumor immunity (46). T_H lymphocytes provide CTLs with growth-stimulating cytokines, prime/activate DCs to effectively present antigen to naive CTL precursors (47-49) and they are important in the development of immune memory (50-52). The development of IgG antibodies to HER-2/neu and the identification of $CD4^+$ T cells that secrete cytokines in response to HER-2/neu peptides or recombinant HER-2/neu protein suggest responses to helper T cells (53-58). We (EC) have identified a promiscuous MHC class II T_H epitope for the HER-2/neu antigen (HER-2_{883}). T cell responses are restricted by HLA-DR1, HLA-DR4, HLA-DR52, and HLA-DR53 (59). Peptide-induced T cells were effective in recognizing naturally processed HER-2/neu protein. The peptide HER-2_{883}, (KVPIKWMALESILRRRF), which was selected by computer algorithm, was

tested for its capacity to stimulate $CD4^+$ T cells isolated from four healthy, MHC-typed individuals (DR1/11, DR1/13, DR4/15, DR7/17) in primary *in vitro* culture using peptide pulsed autologous DCs. T cells that proliferated were found to react with peptide and recombinant HER-2/neu intracellular domain protein presented by autologous DCs (see below). These results, showing reactivity with recombinant protein, suggest that HER-2_{883} is naturally processed, as the peptide stimulated T cells react with DCs primed with recombinant protein. Clearly, HER-2_{883} is a naturally processed peptide epitope and is promiscuous for multiple HLA-DR epitopes, making it an ideal candidate for therapeutic applications.

1.8 Because of the expression of MUC1 and HER-2/neu in multiple cancers, the development of this peptide-based immunotherapy can potentially impact the treatment of multiple disease entities, not only adenocarcinomas but hematopoietic malignancies as well. There is considerable interest in the use of the MUC1 peptide vaccination for treatment of multiple myeloma following transplant when there is minimal residual disease prior to remission.

1.9 GM-CSF

Granulocyte-macrophage colony stimulating factor (GM-CSF) is a commercially available cytokine currently used in patients undergoing chemotherapy to shorten the duration of post-chemotherapy neutropenia. Recently published evidence also suggests that GM-CSF may play a role as an immune adjuvant (60). The following observations illustrate the mechanisms by which GM-CSF can potentiate the immunogenicity of an antigen: 1) GM-CSF is a key mediator of dendritic cell (DC) maturation and function (61); 2) GM-CSF increases surface expression of class I and II MHC molecules as well as co-stimulatory molecules of dendritic cells *in vitro* (61); 3) GM-CSF enhances antibody responses to known immunogens *in vivo* (62); 4) tumor cells transfected with genes encoding/expressing GM-CSF are able to induce long lasting, specific anti-tumor immune responses *in vivo* (63); 5) GM-CSF encapsulated in biodegradable microspheres mixed with whole tumor cells resulted in systemic anti-tumor immune responses comparable to those of GM-CSF transfected tumor cells (64). Therefore, addition of GM-CSF to an oligopeptide antigen may substantially enhance its immunogenicity.

In an attempt to optimally enhance the immunogenicity of the peptides we will deliver the antigens and GM-CSF emulsified in incomplete Freund's adjuvant (IFA, Montanide ISA-51). This delivery mechanism should be comparable to a previously demonstrated delivery mechanism utilizing GM-CSF suspended in microspheres and mixed with tumor cells (antigens). We hypothesize that the emulsified GM-CSF in close proximity to tumor antigen peptides will substantially enhance their immunogenicity. This proximity of antigen and GM-CSF seems to be necessary for the adjuvant effect of GM-CSF, as systemic administration of equivalent doses in animal models has not demonstrated adjuvant activity. Also, the adjuvant/local inflammatory properties of IFA may play a role in attracting antigen presenting cells to the site of injection (54, 65). We have preliminary data demonstrating the plausibility of such a mechanism.

1.10 Preliminary data demonstrating the feasibility of this approach already exists. Rosenberg et al. published effective generation of peptide-specific T cells in melanoma patients immunized with peptides derived from gp100 (66). Despite the demonstration of a specific immune response, no clinical responses were detected. Addition of systemic GM-CSF resulted in more pronounced CTL and delayed type hypersensitivity reactions and in a few cases objective tumor regressions (64). Salgaller et al. utilized a peptide derived from the gp100 epitope suspended in IFA and demonstrated generation of specific T cell responses to the peptide in melanoma patients (67). Both studies suggest that increased immunogenicity of the peptide antigens leads to a more pronounced T cell response, which in some cases results in a clinically relevant anti-tumor effect. In the proposed study, we will combine the immunoadjuvant effects of both IFA and GM-CSF with the goal of increasing the immunogenicity of the MUC1 and HER-2/neu immunodominant peptides.

Preliminary observations in an ongoing clinical study (MC9973) utilizing HLA-A2 specific melanoma differentiation antigen peptide vaccines in which the peptide is emulsified in a suspension of IFA and GM-CSF is demonstrating enhanced skin reactions if peptide emulsified in IFA is administered in the presence of GM-CSF. A dose of 50 μg of GM-CSF in the presence of IFA and peptide results in extensive local skin reactions as well as evidence of a clinical response in one of seven patients thus far.

1.11 CpG

Therapeutic properties of bacteria in the treatment of malignant diseases (i.e. Coley's toxin) is an observation that has permeated the oncology literature for almost a century. More recently, it has been demonstrated that bacterial DNA possesses unique immunomodulatory features of potential utility in cancer therapy. Specifically, unmethylated CpG are able to stimulate NK cells and B cells. Furthermore, synthetic oligodeoxynucleotide (ODN) constructs containing unmethylated CpG motifs (CpG-ODN) were able to activate dendritic cells (DC) enhancing their antigen processing/presentation properties and stimulating production of Th1 cytokines necessary for CTL immune responses. Thus, CpG ODN appeared to function as an immune adjuvant. Several preclinical and clinical works illustrate the ability of CpG-ODN to function as a potent immune adjuvant for various forms of vaccines. One of the more interesting works, pertinent to this study, demonstrates the ability of CpG ODN to induce CTLs against a peptide vaccine when administered in conjunction with incomplete Freund's adjuvant (IFA) (68). These authors used a MART-1/Melan-A_{26-35} peptide emulsified in IFA with or without the addition of 50ug of CpG ODN to immunize human human D^b (HHD) A2 transgenic mice. Their data suggest superior anti-peptide immunization in the CpG-ODN immunized group as determined by the frequency of tetramer positive CTLs. Our own data support these findings demonstrating superior immunization efficacy of IFA+CpG-ODN with ova peptide of C57BL/6 mice when compared to either IFA+peptide or complete Freund's adjuvant (CFA) + peptide (data not shown). An additional benefit to the CpG-ODN adjuvant is that it has been shown to be especially good at enhancing cellular and humoral

immunity and promoting a Th1-type of response in older mice (69). The population that develops cancer is mainly older individuals, thus the CpG-ODN adjuvant may be particularly relevant for this trial. Based on preclinical data suggesting the potent immune adjuvant properties of CpG co-emulsified with peptides in IFA, we elected to test the efficacy of CpG-ODN in the setting of a peptide vaccine immunization in this clinical trial.

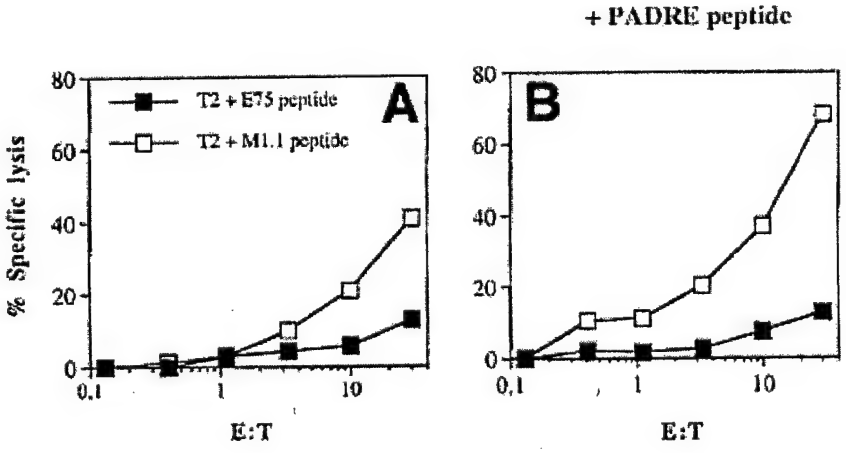
1.12 Preliminary Data

Preliminary data will be presented in multiple sections. First, we will provide data to support the choice of MUC1 and HER-2/erbB2 antigenic epitopes for this trial. Next, we will define our experience using peptides to stimulate tumor reactive T cells for cancer immunotherapy. Finally, we will discuss our experience with the immune adjuvants GM-CS and CpG-ODN. These preliminary data provide a strong foundation for the current proposal.

1.12.1 Identification of CTL Epitopes from MUC1

Using a computer analysis of the MUC1 amino acid sequence, two novel peptides were identified with a high binding probability to the HLA-A2 molecule (34). Two peptides from MUC1 were identified; one from the tandem repeat M1.1 (STAPPVHNV$_{950\text{-}958}$) and one from the leader sequence M1.2 (LLLLTVLTV$_{12\text{-}20}$). The presence of the V in position 6 increases the binding of the M1.1 peptide to the HLA-A2 molecule. There is some variability in the tandem repeats in MUC1 and this sequence is found in the last tandem repeat. Cytotoxic T cells were induced from healthy donors by primary *in vitro* immunization using peptide-pulsed dendritic cells. The peptide-induced CTL lysed tumors endogenously expressing MUC1 in an antigen-specific and HLA-A2-restricted fashion.

CTL.M1.1



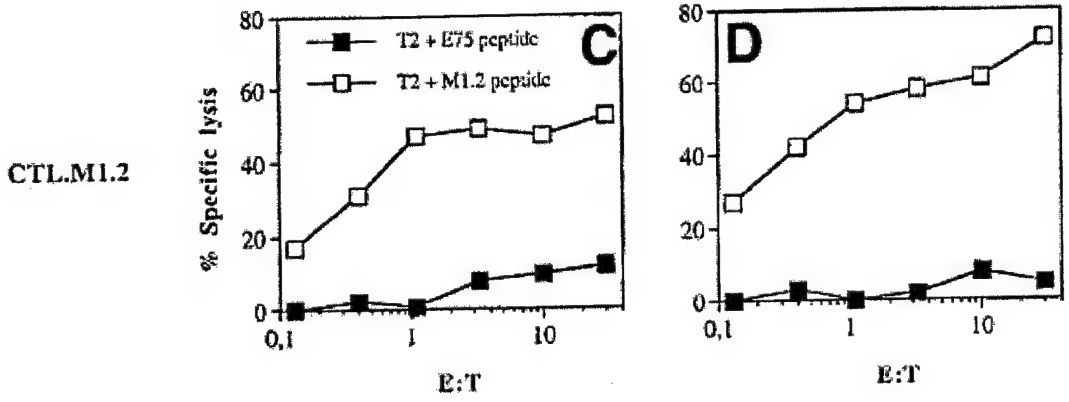


Figure 1. Induction of CTL responses by peptide-pulsed dendritic cells. Adherent peripheral blood mononuclear cells were grown for 7 days with GM-CSF, IL-4, and TNF alpha. DCs pulsed with the synthetic peptides derived from the MUC1 protein (M1.1 and M 1.2) were used to induce a CTL response in vitro. In addition to the MUC1 peptide DCs were incubated with the PAN-DR binding peptide PADRE as a T-helper epitope. Cytotoxic activity of induced CTL was determined in a standard ^{51}Cr-release assay using T2 cells as targets pulsed for 2 hours with 50 µg of the cognate (open symbols) or irrelevant HER-2/neu protein-derived protein derived E75 peptide (solid symbols). (data reproduced from Brossart 1999 (34))

Next, the ability of the induced MUC1-specific CTL lines to lyse tumors expressing MUC1 was tested. MCF-7 cells that express MUC1 endogenously and are HLA-A2 positive were used as targets in a standard ^{51}Cr-release assay. The controls were SK-OV-3 cells, which express MUC1, but are HLA-A2 negative and the immortalized B cell line, Croft, which is A2 positive and was pulsed with MUC1 M1.1 or M1.2 peptides or the irrelevant HER-2/neu E75 peptide.

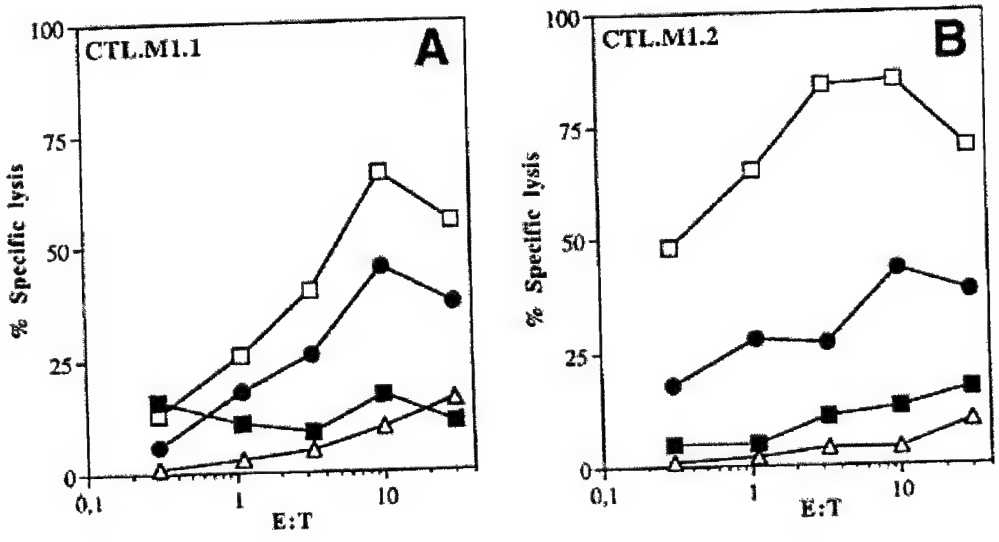
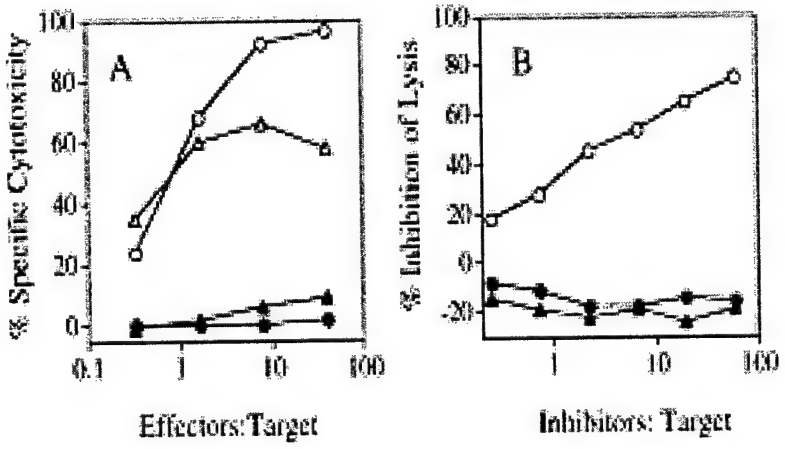


Figure 2. Lysis of cancer cells endogenously expressing MUC1 by CTL.M1.1 (A) and CTL.M1.2 (B). Human breast cancer cell line MCF-7 (HLA-$A2^+$/$MUC1^+$), ovarian cancer cell line SK-OV-3 (HLA-$A2^-$/$MUC1^+$), and the immortalized B-cell line Croft (HLA-$A2^+$/$MUC1^-$) were used as targets in a standard ^{51}Cr-release assay. Croft cells were pulsed with the MUC1 peptides or an irrelevant HER-2/neu-derived peptide E75. (■) Croft + E75 peptide; (□) Croft + M1.1 (A) or M1.2 (B); (●)MCF-7; (Δ) SK-OV-3.

We have chosen to use the M1.1 peptide based on the large amount of data on the response to the MUC1 tandem repeat peptide, both in the human situation as well as in the mouse. Obviously only the human data are relevant for the clinical trials. We will use a HER-2/neu helper epitope (see below, not the PADRE helper epitope)

In the case of HER-2/neu, we have identified a novel CTL epitope HER-2 (9_{435}), which bound HLA-A2.1 with intermediate affinity (IC_{50} 74.6 nM). The peptide identified is: ILHNGAYSL. The .221(A2.1) cell line, produced by transferring the HLA-A2.1 gene into the HLA-A, -B, -C null mutant human lymphoblastoid cell line .221, was used as target (peptide loaded) to measure activity of HLA-A2.1 restricted CTL (70). The CTLs elicited following in vitro stimulation effectively killed HLA-$A2.1^+$ tumor cells, showing that the antigen is appropriately processed by tumors (Fig. 3A). In addition, recognition of the tumor cell target was significantly inhibited by unlabeled (cold) target pulsed with HER-2 (9_{435}) peptide, but not by unlabeled targets either unpulsed or pulsed with a control HLA-A2.1 binding peptide (Fig. 3B).



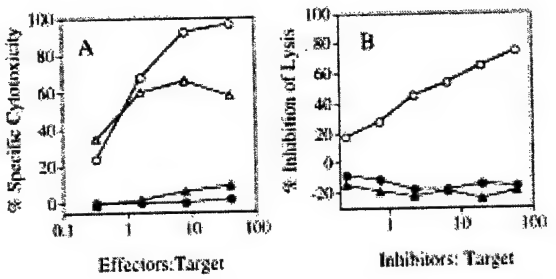


Figure 3. HER-2(9_{435}) specific CTL can kill tumor cells. (A) HER-2(9_{435}) specific CTL were used as effector cells to test for the lysis of the following target cell lines: o, .221A2.1 pulsed with HER-2(9_{435}); •, .221A2.1 without peptide; Δ, SW403 (colon CA, $A2^+$, HER-2/neu^+); ▲, HT-29 (colon ca, $A2^-$, HER-2/neu^+). (B): Antigen specificity demonstrated by cold target inhibition assay. Lysis of ^{51}Cr labeled SW403 cells at an effectors/target ratio of 10:1 by the HER-2(9_{435}) specific CTL was blocked at various Inhibitors/Target ratios by the following cold targets: o, .221A2.1 pulsed with HER-2(9_{435}); ▲, .221A2.1 pulsed with irrelevant A2.1 binding peptide (HBc_{18-27}); •, .221A2.1 without peptide.

In addition to the class I epitopes described above, we (EC) have defined a promiscuous MHC class II epitope for HER-2/neu using the algorithm tables published by Southwood et al. (59, 67). The epitope identified is HER-2_{883} (KVPIKWMALESILRRRF). It is important to show that these peptides represent true T cell epitopes that are relevant for the development of tumor immunotherapy. For these experiments autologous PBMCs or DCs were used as APCs and recombinant DNA derived intracellular domain or extracellular domain protein fragments of HER-2/neu were used as a source of antigen. The data in Fig. 4 show that four HER-2_{883}-reactive T cell lines proliferated well to HER-2/neu intracellular domain protein, which encompasses the HER-2_{883} peptide but not to HER-2/neu extracellular domain (ECD), which lacks HER-2_{883}.

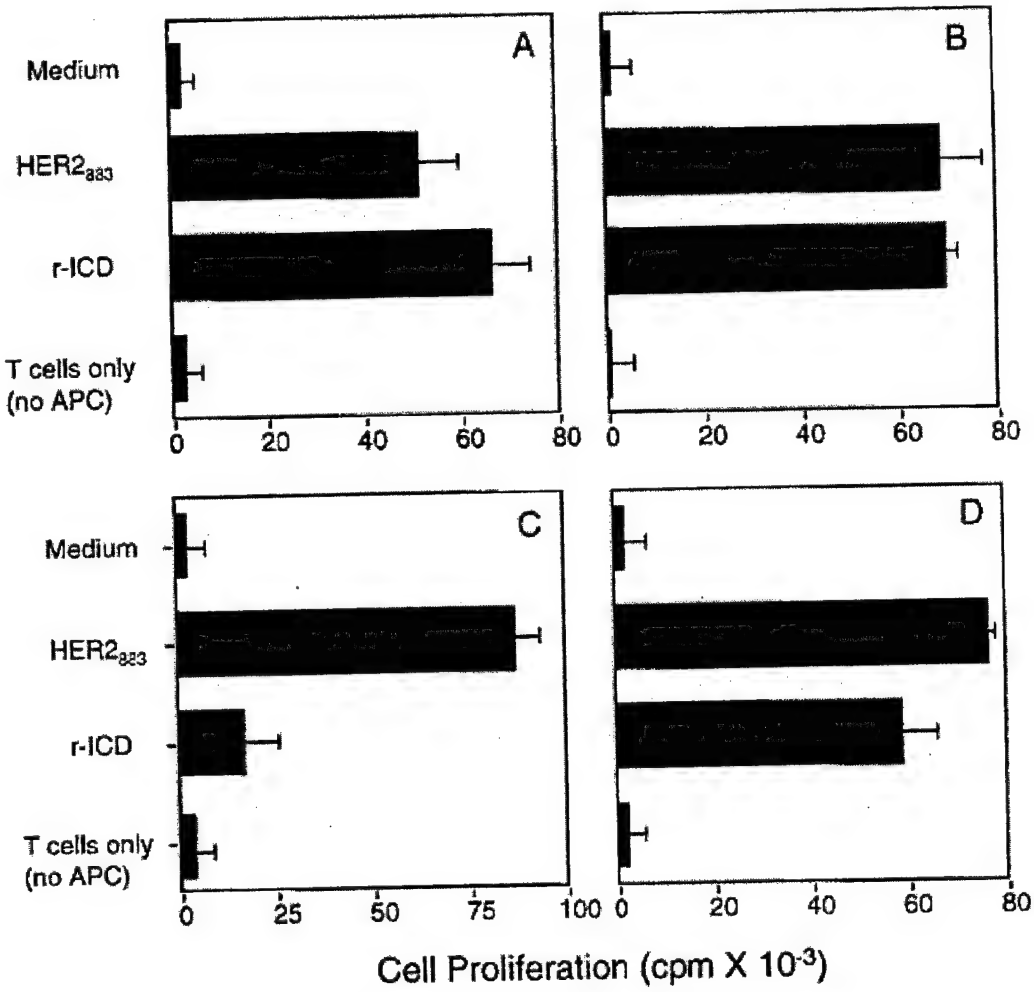


Figure 4. HER-2_{883}-specific CD4+ T cells can recognize recombinant HER-2/neu intracellular domain (*r-ICD*) protein presented by autologous Dcs in the context of several HLA-DR alleles. The HER-2_{883}-reactive HTLs, TCL-7C (panel *A*, HLA-DR53 restricted), TCL-6D (panel *B*, HLA-DR4-restricted), a clone of TCL-1D (panel *C*, HLA-DR52-restricted), and TCL-1E (panel *D*. HLA-DR53 restricted), were tested for their capacity to proliferate to autologous DCs in the presence of HER-2_{883} peptide (2.5 mg/ml) or recombinant HER-2/neu recombinant ICD protein (10 mg/ml). No significant proliferative response was observed against HER-2/neu ECD protein (data not shown). Values shown are the means of triplicate determinations; *bars*, SD.

2.0 Goals

2.1 To determine the safety and immunization efficacy of two doses of MUC1 and HER-2/neu peptide vaccines combined with CpG or GM-CSF as immune adjuvants suspended in montanide ISA-51.

2.2 To describe the impact of immunization on clinical outcomes in patients with MUC1 positive breast cancer.

3.0 Patient Eligibility

Prior to discussing protocol entry with the patient, call the Randomization Center to insure that a place on the protocol is open to the patient.

3.1 Inclusion criteria

3.1.1 Age ≥18 years.

3.1.2 Completed "standard therapy" for breast cancer (at least 3 months prior to registration) and currently with no evidence of disease.

3.1.3 Histologically confirmed adenocarcinoma of the breast treated with surgery, adjuvant chemotherapy, and/or radiation therapy

3.1.4 No radiographic evidence of disease at the time of registration.

3.1.5 MUC1 positive breast cancer by central review.

3.1.6 HER-2 positive breast cancer defined as HER-2 gene amplified by FISH or HER-2 expression of 1+, 2+ or 3+ by HercepTest on central review.

3.1.7 HLA-A2 positive.

3.1.8 Laboratory values obtained ≤14 days prior to registration:

- Hemoglobin ≥8.0 g/dL
- Platelets ≥75,000/uL
- ANC ≥1,500/uL
- Creatinine ≤2 ULN
- AST ≤ 2 ULN

3.1.9 Capable of understanding the investigational nature, potential risks and benefits of the study and capable of providing valid informed consent.

3.1.10 Willingness to return to Mayo Clinic Rochester/Scottsdale/Jacksonville for treatment and study-related follow up.

3.1.11 Life expectancy ≥12 weeks.

3.1.12 Willingness to provide the blood and tumor specimens and complete the imaging studies as required by the protocol.
Note: The goals of this study include assessment of the biologic effects on surrogate markers of the agent(s) being tested and are, therefore, contingent upon availability of the blood and tumor specimens and completion of the required imaging studies.

3.2 Exclusion criteria

3.2.1 ECOG performance status (PS) 3 or 4 (see Appendix III).

3.2.2 Uncontrolled infection.

3.2.3 Any of the following:
- Known HIV infection
- Other circumstances (i.e. concurrent use of systemic immunosuppressants and immunocompromising condition) that in the opinion of the physician renders the patient a poor candidate for this trial

3.2.4 Any of the following prior therapies:
- Chemotherapy ≤ 6 weeks prior to registration
- Immunotherapy ≤4 weeks prior to registration
- Biologic therapy ≤4 weeks prior to registration *(concurrent anti-estrogen therapy is allowed)*
- Radiation therapy ≤6 weeks prior to registration

3.2.5 Failure to fully recover from acute, reversible effects of prior breast cancer therapy regardless of interval since last treatment.

3.2.6 Any of the following:
- Pregnant women
- Nursing women unwilling to stop breast feeding
- Women of childbearing potential who are unwilling to employ adequate contraception (condoms, diaphragm, birth control pills, injections, intrauterine device [IUD], or abstinence, etc.)

NOTE: This study involves an investigational agent whose genotoxic, mutagenic and teratogenic effects on the developing fetus and newborn are unknown.

3.2.7 Other concurrent chemotherapy, immunotherapy, radiotherapy, or any ancillary therapy considered investigational (utilized for a non-FDA-approved indication and in the context of a research investigation).

3.2.8 Disease-free of prior invasive malignancies for at least 5 years (with the exception of curatively-treated basal cell or squamous cell carcinoma of the skin or carcinoma in situ of the cervix

4.0 Test Schedule

Tests and procedures	≤14 days prior to registration	Prior to each subsequent treatment (q 4 weeks)	Long term f/u q3 months for 2 years
History and exam, weight, PS	X	X	X
Height	X		
Hematology group: WBC, ANC, Hgb, PLT	X	X	X
Chemistry group: total and direct bilirubin, AST, creatinine.	X	X	X
HLA class I and II typing[R]	At any time prior to registration		
Serum pregnancy test[1]	X		
Tumor typing[R]	At any time prior to registration		
Tumor evaluation by imaging study (x-ray, CT or PET)[R,2]	X	Every other cycle	X
DTH skin testing (common recall antigens)[3, R]	X	Prior to cycle 6 only	
Research blood specimens	X	X[4]	X[4]

1. Serum pregnancy test obtained ≤7 days prior to registration.
2. Research funding for imaging studies will be provided in cases where f/u imaging is not part of "standard of care".
3. DTH skin testing will be performed using the same complement of antigens in routine use at the treatment site.
4. Research blood samples will be performed before registration as well as prior to cycles 2, 4 and 6 of therapy as well as every other cycle of long term follow-up starting at 3 months after completion of Rx.

5.0 Stratification Factor

Adjuvant chemotherapy: yes versus no
Ongoing anti-estrogen therapy: yes vs no.

6.0 Registration/Randomization Procedures

6.1 To register a patient, call (4-2753) or fax (4-0885) a completed eligibility check-list to the Randomization Center between 8 a.m. and 5 p.m. central time Monday through Friday.

6.2 A signed HHS 310 form must be on file in the Randomization Center before an investigator may register any patients.

6.3 Randomization Center personnel will check patient eligibility and the existence of a signed consent form before a patient will be registered into this study.

6.3.1 Patient has given permission to store sample(s) for future research of melanoma:

6.3.2 Patient has given permission to store sample(s) for future research to learn, prevent, or treat other health problems:

6.3.3 Patient has given permission to give their sample(s) to outside researchers:

6.4 Treatment on this protocol must commence at Mayo Clinic under the supervision of a medical oncologist.

6.5 Treatment cannot begin prior to registration and must begin *3 days after registration.

6.6 Pretreatment tests must be completed within the guidelines specified on the test schedule.

6.7 All baseline symptoms must be documented and graded on the oncology record.

7.0 Protocol Treatment

7.1 As part of the registration process described in Section 6.0, the Mayo Clinic Cancer Center (MCCC) Randomization Center will assign patients to arms 1 through 4

7.2 Treatment Schedules:

Arm A

Arm	Agent		Dose	Route	RxDays	ReRx
A	Montanide ISA-51		1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1		500ug			
	HER-2 peptide A		500ug			
	HER-2 peptide B		500ug			
	GM-CSF		100ug			

Arm B

Arm	Agent		Dose	Route	RxDays	ReRx
B	Montanide ISA-51		1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1		1000ug			
	HER-2 peptide A		1000ug			
	HER-2 peptide B		1000ug			
	GM-CSF		100ug			

Arm C

Arm	Agent		Dose	Route	RxDays	ReRx
C	Montanide ISA-51		1.2mL	subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1		500ug			
	HER-2 peptide A		500ug			
	HER-2 peptide B		500ug			
	CpG		XX			

Arm D

Arm	Agent	Time	Dose	Route	RxDays	ReRx
D	Montanide ISA-51		1.2mL	Subcutaneous injection in un-dissected LN region	Day 1 of Week 1	Q4 weeks (28 days) x 6 cycles
	MUC1		1000ug			
	HER-2 peptide A		1000ug			
	HER-2 peptide B		1000ug			
	CpG		XX			

7.3 Ten patients will be randomly assigned to receive one of the four treatment schedules. Doses will not be escalated in any individual patient.

Vaccines will be prepared in a single vile and administered as multiple (2-3) subcutaneous injections in a single draining regions of un-disturbed axillary or inguinal lymh nodes. Injection sites should be rotated with different treatment cycles.

8.0 Dosage Modification Based on Adverse Events - Adjustments are based on adverse events observed since the prior dose.

ALERT: *ADR reporting may be required for some adverse events. See Section 10.*

→ → *Use Common Toxicity Criteria (CTC) Version 2.0 unless otherwise specified* ← ← *AT SCHEDULED RE-TREATMENT*			
CTC CATEGORY	ADVERSE EVENT	AGENT	TREATMENT ADJUSTMENT or OTHER ACTION
ALLERGY/ IMMUNOLOGY	≥Grade 2 allergic reaction/hypersensitivity	Montanide GM-CSF CpG Peptides	Discontinue vaccinations and begin observation.
	≥Grade 2 autoimmune reaction (excluding vitiligo)		Discontinue vaccinations and begin observation.
ALL OTHERS	≥Grade 3 Hematologic or≥Grade 4 Nonhematologic (excluding alopecia)	Montanide GM-CSF CpG Peptides	Discontinue vaccinations and begin observation.

9.0 Ancillary Treatment/Supportive Care

9.1 Patients should receive full supportive care while on this study. This includes blood product support, antibiotic treatment and treatment of other newly diagnosed or concurrent medical conditions. All blood products and concomitant medications such as antidiarrheals, analgesics, anti-emetics received from the first administration of study drugs until 30 days after the final dose are to be recorded in the medical record.

9.2 Patients participating in this clinical trial are not to be considered for enrollment in any other study involving a pharmacologic agent (drugs, biologics, immunotherapy approaches, gene therapy) whether for symptom control or therapeutic intent.

10.0 Adverse Event (AE) Reporting and Monitoring

10.1 This study will utilize the Common Toxicity Criteria (CTC) version 2.0 for adverse event monitoring and reporting. The CTC version 2.0 can be downloaded from the CTEP home page (http://ctep.info.nih.gov/CTC3/ctc_ind_term.htm). All appropriate treatment areas should have access to a copy of the CTC version 2.0.

10.1.1 Adverse event monitoring and reporting is a routine part of every clinical trial. First, identify and grade the severity of the event using the CTC. Next, determine whether the event is expected or unexpected (refer to Section 15.0 and/or product literature) and if the adverse event is related to the medical treatment or procedure (see Section 10.12). With this information, determine whether an adverse event should be reported as an

expedited report (see Section 10.2) or as part of the routinely reported clinical data.

Expedited adverse event reporting requires submission of a written report, but may also involve telephone notifications. Telephone and written reports are to be completed within the timeframes specified in Section 10.2. All expedited adverse event reports should also be submitted to the local Institutional Review Board (IRB).

10.1.2 Assessment of Attribution

When assessing whether an adverse event is related to a medical treatment or procedure, the following attribution categories are utilized:

Definite - The adverse event *is clearly related* to the investigational agent(s).

Probable - The adverse event *is likely related* to the investigational agent(s).

Possible - The adverse event *may be related* to the investigational agent(s).

Unlikely - The adverse event *is doubtfully related* to the investigational agent(s).

Unrelated - The adverse event *is clearly NOT related* to the investigational agent(s).

10.2 Expedited Adverse Event Reporting Requirements

Phase I, II and III Studies (Investigational)

	Grade 4 or 5[1] Unexpected with Attribution of Possible, Probable, or Definite	Other Grade 4 or 5 or Any hospitalization during treatment[6]	Secondary AML/MDS[2]
Call Cancer Center Pharmacist[3] within 24 hours	**X**		
Submit written report within 5 working days[4]	**X**		
NCI/CTEP Secondary AML/MDS Report Form within 15 working days[5]			**X**
Submit Grade 4 or 5 Non-AER Reportable Events/Hospitalization Form within 5 working days.[6]		**X**[6]	

1. Includes all deaths within 30 days of the last dose of investigational agent regardless of attribution or any death attributed to the agent(s) (possible, probable, or definite) regardless of timeframe.
2. Reporting for this AE required during or after treatment.
3. Contact the Cancer Center Pharmacist (Mayo Clinic - Rochester) at (507) 284-2701.
4. Use *Adverse Event Expedited Report – Single Agent* or *Multiple Agents* report form. Submit to the Cancer Center Pharmacist (Mayo Clinic - Rochester) and to the Cancer Center Protocol Development Coordinator (PDC) for IRB reporting. The pharmacist will report to the Food and Drug Administration (FDA) as required.
5. Submit per form-specified instructions and provide copy to Cancer Center Pharmacist and Cancer Center PDC. The PDC will facilitate IRB as warranted by the event.
6. In addition to standard reporting mechanism for this type of event, submit information to the PDC. The PDC will facilitate IRB reporting as warranted by the event. If Adverse Event Expedited Report – Single Agent or Multiple Agents report form was completed, this form does not need to be completed.

10.3 Adverse events to be graded at each evaluation and pretreatment symptoms/conditions to be evaluated at baseline per Common Toxicity Criteria (CTC) Version 2.0 grading unless otherwise stated in the table below:

CTC Category	Adverse event/Symptoms	Baseline	Each evaluation
Constitutional Symptoms	Fatigue	X	X
Dermatology/Skin	Injection site reaction		X
	Rash/desquamation	X	X
Pain	Arthralgia	X	X
	Myalgia	X	X
	Bone pain	X	X

10.3.1 Documentation of the following AEs, when experienced by a participant but not specified in Section 10.3, are to be submitted using the case report forms:

10.3.1.1 Grade 1 and 2 AEs deemed possibly, probably, or definitely related to the study treatment or procedure.

10.3.1.2 Grade 3, 4, or 5 AEs and deaths within 30 days of the participant's last treatment, regardless of attribution to the study treatment or procedure, with the exception of signs or symptoms definitely related to the participant's disease or disease progression.

10.3.1.3 Any death more than 30 days after the participant's last study treatment or procedure that is felt to be at least possibly treatment related must also be submitted as a Grade 5 AE, with a CTC type and attribution assigned.

11.0 Treatment Evaluation

11.1 For the purposes of this study, patients should be re-evaluated every 8 weeks.

11.2 At the time of reevaluation, patients will be classified in the following manner:

11.2.1 No evidence of disease (NED).

11.2.2 Breast cancer recurrence (REC). Local/regional breast cancer recurrence is defined as the development of tumor (except LCIS) in the ipsilateral breast (after lumpectomy); in the soft tissue/chest wall and/or skin of the ipsilateral chest wall; or tumor in the ipsilateral internal mammary, infraclavicular, or axillary nodes or soft tissue of ipsilateral axilla. Suspected tumor recurrence in the ipsilateral breast, chest wall structures or lower (level I ± II) axillary nodal areas must be confirmed by biopsy or cytology. Histologic or cytologic confirmation of tumor is recommended for internal mammary or infraclavicular/high axillary nodal recurrence. A distant recurrence is defined as development of tumor in areas other than the local/regional area that is documented by a positive cytology aspirate, biopsy, or imaging studies.

11.2.3 New primary (NEWP): A new primary is defined as the development of contralateral breast cancer or a second cancer other than squamous or basal cell carcinoma of the skin, carcinoma in situ of the cervix or LCIS of the breast that is histologically confirmed.

11.3 Further treatment after the documentation of a breast cancer recurrence or second primary cancer is left to the discretion of the treating physician.

12.0 Descriptive Factors - None

13.0 Treatment/Follow–up Decision at Evaluation of Patient

13.1 Patients who have not recurred at time of their reassessment and have <u>not</u> experienced intolerable toxicity may continue protocol treatment at the same dose level for a maximum of 6 cycles or until progression of disease, a second primary or an intolerable adverse event occurs.

13.2 Patients who develop progression of disease, a second primary or intolerable toxicity will be removed from protocol treatment and go to the event monitoring phase of the study. Subsequent treatment is at the discretion of the treating physician.

13.3 Patients may refuse further protocol treatment at any time and go to the event-monitoring phase of the study.

13.4 If a patient is declared ineligible by the study team, on-study material, treatment evaluation forms, and end of treatment form should be submitted. No further follow-up after notification of ineligibility is required.

13.5 If a patient is declared a cancel by the study team, on-study material should be submitted. No further follow-up is required.

14.0 Correlative/Translational Studies

14.1 Description of Assays

Active vaccines for the immunotherapy of solid tumors have met with only limited success. It is our hypothesis that the causes of this failure are multifactorial and can be improved by the inclusion of stringent patient selection criteria, careful dose titration based on immunologic response monitoring, and correlation of immunologically based dosing parameters with clinical outcome. The following sections define the strategies that will be employed in this trial to evaluate immunologic response to MUC1, and HER-2 peptides.

14.1.1 Immune Responses to T Helper and CTL Epitopes

14.1.1.1 Elispot

Estimates of frequencies of peptide-specific, IFNγ- and IL-5-producing cytotoxic T lymphocytes and helper T lymphocytes will be obtained by ELISPOT assays following *in vitro* stimulation with peptide-sensitized stimulator cells [87, 89]. IL-5 production, rather than that of IL-4, will be assayed because of the increased signal:noise ratio [89]. CD8+ and CD4+ T cells will be positively selected by magnetic activated cell sorting (MACS, Miltenyi Biotech) from cryopreserved and thawed peripheral blood lymphocyte buffy coat. Antigen-presenting cells (APCs) will also be isolated from CD4-/CD8- cell population by MACS (beads and reagents purchased from Miltenyi Biotech). CD8+ and CD4+ responder T cells will be stimulated with irradiated APCs pulsed with the target peptides used for vaccination. After 5 days of co-culture, the responding cells will be diluted, titrated, and re-stimulated with APCs pulsed with target peptides for 24 hours in 96 well microtiter ELISPOT plates coated with IFNγ- or IL-5-specific capture antibody (ELISPOT Kit purchased from MABTECH, Stockholm, Sweden). The target peptides for re-stimulation include the peptide used for primary stimulation (MUC1 and HER-2 peptides) and a negative control peptide (YIGEVLVSV). The wells are washed and treated with ALP-conjugated secondary antibody and cytokine-producing spots

detected using appropriate substrate (all reagents are provided in the kit). After stopping the reaction, the developed microtiter plates are shipped to Zellnet Consulting in New York for evaluation of number of spot-producing cells for each responder cell titration. All analyses are performed by the consulting firm and data provided electronically to the investigator. The difference between the frequency of spot-producing cells obtained with the target peptides and control peptide will determine the frequency of peptide-specific, cytokine-producing CD4+ or CD8+ T cells.

14.1.1.2 Dimers

The estimation of frequencies of CTLs that recognize specific peptides bound to class I molecules became increasingly easier and more quantifiable with the construction and application of class I tetramers (12). However, we have found that this system has a number of significant drawbacks in real-world application. Therefore, we propose to replace tetramers in our studies with commercially available class I dimers. Briefly, class I dimers are comprised of mouse IgG1 molecules fused to class I heavy chains from which the transmembrane and cytoplasmic tail portions have been removed (71). They are complexed with mouse β2M and are produced as secreted molecules in murine myeloma cells. Since they are produced in murine cells, they are properly glycosylated and have incorporated an array of endogenous peptides into their binding sites. Dimers of HLA-A2 molecules are commercially available (BD Biosciences); the only step required before use is overnight incubation with an excess of the experimental peptide to replace the bound, endogenous peptides. An aliquot of 5ug HLA-A2 dimers is mixed with a 640x molar excess of experimental peptide dissolved in dimethyl sulfoxide (DMSO) and incubated overnight at 4°C. On the day of staining, test PBLs are thawed, washed, and resuspended in the manufacturer's recommended staining buffer at 10 x 106 cells/ml. Peptide-exchanged dimers are added to 50ul cell volumes and incubated for 60 min at 4°C. This cell suspension is then stained with PE-anti-mouse IgG1, FITC-anti-CD8, and APC-anti-CD3 antibodies for 30 min at room temperature. The stained cells are analyzed with FACSCAN instrumentation and CellQuest software (BD Biosciences); a minimum of 5 x 105 cells/sample are analyzed for accurate estimation of CTLs with low frequencies. The analysis involves (1) gating on lymphocytes using forward and side-scatter; (2) gating on APC-positive PBLs that stain with anti-CD3, and (3) analyzing

the gated cells for PE and FITC staining. The frequency of doubly stained cells (dimer+/CD8+) will be estimated for each of three replicate wells for calculation of the mean frequency (±s.e). All assays will include a negative control dimer that includes the YIGEVLVSV from the HA-2 human minor histocompatibility antigen (72).

14.1.2 Antigenic Profiling

14.1.2.1 Expression of Class I HLA Antigens on tumor tissue.

Initial entry criteria require HLA-A typing of peripheral blood with subsequent confirmation of HLA class I antigen expression on tumor cells by immunohistochemistry. One of the mechanisms by which tumors are postulated to evade the immune response is by down regulation of classical HLA molecules necessary for antigen presentation.

14.1.2.2 Tumor Expression of MUC1 and HER-2

Tumor blocks will be used to determine the levels of expression of HER-2 and MUC1 on breast cancer tumor cells obtained at the time of most recent surgical resection. HER-2 expression will be determined using a clinical grade test +1 to +3 and levels of expression will be graded on a semi-quantitative scale. MUC1 expression will be determined by positive staining with one of three antibodies to MUC1 (HMFG-2, BC-2, or B27.29). Negative controls will be incubated with PBS instead of monoclonal antibody. Staining of cytoplasm and plasma membrane will be evaluated. Cells will be considered positive when at least one of these components is stained. Antibody staining patterns will be scored in a semi quantitative manner from +1 to +3.

14.1.3 Sample Schedule

14.1.3.1 Blood

100 mL of blood will be collected (heparin) prior to registration and prior to each subsequent immunization as well as every 3 months after conclusion of active therapy until 24 months following registration.

14.1.3.1 Tumor

Tumor blocks will be collected from the patient's most recent surgery prior to study registration.

14.1.4 Sample Preparation

14.1.4.1 Blood

Peripheral blood lymphocytes (PBLs) are enriched by flotation over Ficoll-Hypaque and frozen in aliquots in 10% DMSO for storage at -150°C. Percentages of CD4+ and CD8+ T cells, B cells, monocytes, and dendritic cells are estimated by flow cytometry with a panel of specific monoclonal antibodies. In addition, proliferation assays (3H-thymidine uptake) are performed to estimate T cell responses to polyclonal stimulus (phytohemagglutinin), target antigens (MUC1 and HER-2/neu) and a recall antigen (tetanus toxoid). These two sets of experiments are important for estimating the representation of individual lymphoid populations and evaluating overall T cell responsiveness. CD8+ (CTLs) and CD4+ (HTLs) are positively purified from cryopreserved and thawed PBLs by magnetic bead separation (Miltenyi Biotek). Additionally, serum will be collected and stored from each of these samples. Cells will then be frozen and stored at -150° for future use.

14.3 Delayed-type hypersensitivity (DTH) skin testing

Skin testing (baseline - prior to registration) will be coordinated through the Mayo Immunization/Allergy Clinic (L-15). A typical panel includes candida, mumps, PPD, and trichophyton. Other antigens may be substituted in the event of antigen unavailability. Patients will return for 1-2 follow-up measurements consistent with L-15 procedures. Patients must have a "positive" reaction to at least one of the antigens tested, to be considered eligible for participation. Patients with only "doubtful" or "negative" reactions will not be considered eligible.

15.0 Drug Information

15.1 MUC1 - *Investigational supply*

15.1.1 Other Names: epithelial membrane antigen (EMA), polymorphic epithelial antigen (PEM), DF3 antigen, Ca1, MAM-6, H23, episialin

15.1.2 Formulation and Storage: to be determined

15.1.3 Drug Procurement and Accountability: to be purchased from Clinalfa

15.2 HER-2 Peptide - *Investigational supply*

15.2.1 Other Names: erbB2, neu

15.2.2 Formulation and Storage: to be determined

15.2.3 Drug Procurement and Accountability: to be purchased from Clinalfa

15.3 Montanide ISA-51 Adjuvant [MONTAN] - *Investigational supply*

15.3.1 Formulation and Storage

Montanide ISA-51 is an oil-based adjuvant product similar to Incomplete Freund's Adjuvant. Which when mixed with a water-based solution on 1:1 w/w ration, forms a water-in-oil emulsion. It consists of highly purified oil, Drakol VR, and a surfactant, mannide oleate. Montanide ISA-51 is manufactured by Sepic, Inc., and is provided in amber glass ampoules containing 3 mL of the solution. Montanide ISA-51 will be purchased from Seppic Inc.

15.3.2 Mode of Action

Acts to enhance immune response to vaccination; the precise mode of action is unknown.

15.3.3 Storage and Stability

The solution is stored at controlled room temperature. Exposure to cold temperatures may result in a clouded solution, which should be discarded. An expiration date is printed on the ampoule label.

15.3.4 Compatibilities/Incompatibilities

The oil may break down the rubber tip of the plunger on syringes; it is advisable to use a different syringe for each ampoule. Do not allow the Montanide ISA-51 to be in direct contact with the rubber tip of the plunger for more time than is necessary to withdraw the solution and inject it into the peptide vial. Fresh syringes will be needed to withdraw the emulsified vaccine from the vaccine vial. Once the emulsion is made, there is less interaction of the oil directly with the rubber tip of the plunger.

15.3.5 Drug Procurement and Accountability

Montanide ISA-51 will be purchased from Seppic Inc. The Cancer Center Pharmacy Shared Resource will store the drug and maintain records of inventory and disposition of all agent received.

15.4 GM-CSF (sargramostim, Leukine®)

15.4.1 Preparation and Storage

For this protocol, the lyophilized version of Leukine® will be used. Each vial contains 250 mcg of sargramostim. Aseptically reconstitute a 250 mcg vial with 1 mL of Sterile Water for Injection, USP. During reconstitution, the diluent should be directed at the side of the vial and the contents gently swirled to avoid foaming. Do not agitate or shake. The reconstituted Leukine® solution is clear, colorless, isotonic with a pH of 7.4 +/- 0.3, and contains 250 mcg/mL of sargramostim. When reconstituted in this manner, the solution contains no preservatives and the product should be used within eight hours.

15.4.2 Known Potential Toxicities

Fever, chills, asthenia, malaise, rash, peripheral edema, dyspnea, headache, pericardial effusion, bone pain, arthralgia, and myalgia.

15.4.3 Drug Procurement:

Leukine 250 mcg vials are available commercially. Drug will be purchased using study grant funds (i.e. Patients will not be charged for GM-CSF).

15.5 CpG

15.5.1 Preparation and Storage: to be determined

15.5.2 Known potential toxicities:

15.5.3 Drug Procurement: to be purchased from Coley Pharmaceutical Group Inc.

15.6 Vaccine Preparation Instructions

15.6.1 General Vaccine Preparation Information

Emulsify the peptide(s)/GM-CSF or CpG mixture with Montanide ISA-51. Prepare the vials as directed for each group below. Place the vial upside down in a tube platform holder of a vortex machine and vortex at highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Because neither the peptide solution nor the Montanide ISA-51 contains preservatives or bacteriostatics, the prepared peptide vaccines should be administered as soon as possible.

15.6.1.1 Arms A and B

Remove the peptide vials from the freezer and thaw at room temperature. Once thawed, add the correct amount peptide (based on the dose level in section 7.11) to a sterile vial with a Teflon-coated stopper. Reconstitute a vial of GM-CSF (sargramostim) 250 mcg, with 1 mL of sterile water for injection yielding a concentration of 250 mcg/mL. Add the correct amount of this solution (100 mcg/0.4 mL/m^2) to the peptide-containing vial. Add 1.2 mL of Montanide ISA-51 to the peptide vial. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two or three tuberculin syringes with equal volumes of this emulsion prior to use). Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused GM-CSF and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

Arms C and D.
Remove the peptide vials from the freezer and thaw at room temperature. Once thawed, add the correct amount peptide (based on the dose level in section 7.11) to a sterile vial with a Teflon-coated stopper. Reconstitute a vial of CpG-ODN (per package insert). Add the correct amount of CpG to the peptide-containing vial. Add 1.2 mL of Montanide ISA-51 to the peptide vial. Place the vial upside down in a tube platform holder of a vortex machine and vortex at the highest speed for 12 minutes. This will minimize the amount of emulsion adhering to the inside surface of the vial. Load two or three tuberculin syringes with equal volumes of this emulsion prior to use). Correct emulsification will be tested by carefully placing a small droplet of the emulsion on the surface of ice-cold distilled water (in a small 10 mL beaker) and observing that the droplet does not disperse after 2 minutes. Discard unused CpG and peptide solution. Each syringe will be identified with the patient's name and confirmed by a second pharmacist. The nurse will administer the vaccine mixture to the patient as soon as possible.

15.7 Vaccine Administration Information

15.7.1 Dose Specifics

Each peptide vaccine will consist of a total volume of approximately 3 to 4 mL, containing the correct dose of the peptide(s), Montanide ISA-51, and GM-CSF or CpG. Be sure to confirm the proper cohort and dose level before preparing the product. Refer to section 7.11, 7.12, and 7.13 for correct dosing level within the proper cohort.

15.7.2 Administration

Vaccinations will be given on day 1 of each treatment cycle. Due to the large volume, each peptide vaccine is administered in 2 to 3 shots in a contiguous location the peptide vaccine should be injected in the vicinity of one of the major nodal basins. This basin must not have been dissected.

15.8 Vaccine Side Effects:

15.8.1 Because of the low dose of GM-CSF used and the slow release nature of the vaccine emulsion, side effects normally seen with systemic treatment doses of GM-CSF should not play a factor in this vaccination treatment. Expected side effects are related to the peptides and Montanide ISA-51. It is possible that the GM-CSF may potentiate the reaction seen at the injection site.

15.8.2 Dermatology/Skin: Injection site reaction, rare granuloma formation, possible development or worsening of pre-existing vitiligo, rash.

15.8.3 Hepatic: transient rises in liver transaminases.

15.8.4 Constitutional: Low-grade fever.

16.0 Statistical Considerations and Methodology

16.1 Study design: This is a pilot study designed to determine the immunologic effects of two dose schedules of a MUC1/HER-2/neu peptide based tumor vaccine in the presence of the immune adjuvant GM-CSF suspended in montanide ISA-51 as well as the immunologic effects of the same dose schedules of the MUC1/HER-2/neu peptide based tumor vaccine in the presence of the immune adjuvant CpG suspended in montanide ISA-51.

16.1.1 A secondary goal of the study is to determine the toxicity profile of each treatment combination

16.2 The study design chosen for this proposal is a stratified randomized design with type of dominant disease (see section 5.0) as the sole stratification factor. Toxicities will be carefully monitored and accrual will be suspended if 2 or more of the first six patients experience a grade 4 hematologic toxicity lasting for 5 or more days. In the event of at least two patients experiencing immunologic toxicity ≥ grade 2 or any toxicity ≥ grade 3 accrual will be temporarily suspended for the given treatment arm.

16.3 Accrual: Ten patients with MUC1/HER-2 positive breast cancer with no evidence of disease will be randomized to each one of the 4 treatment schedules. The total number of eligible patients to be accrued will be 40. Patients will be allocated to each treatment schedule using a dynamic allocation procedure that balances the marginal distribution of type of dominant disease between treatment combinations. The expected accrual rate for this study is about 15-20 patients at Mayo Clinic Rochester and about 5-7 patients each at Mayo Clinic Scottsdale and Mayo Clinic Jacksonville per year.

16.4 Study Endpoints:

16.4.1 Primary Endpoints

16.4.1.1 The immunologic parameters of interest are: (1) the percentage of CD4+ T cells, CD8+ T cells, B cells, monocytes, and dendritic cells in a patient's peripheral blood sample as estimated by flow cytometry with a panel of monoclonal antibodies and (2) the frequency of both peptide-specific IFN-gamma producing T cells and peptide-specific IL-5 producing T cells estimated by ELISPOT assays following *in vitro* stimulation with peptide-sensitized stimulator cells for the MUC1 and HER-2 peptides.

16.4.1.2 The number and severity of hematologic and non-hematologic toxicities reported using the NCI-CTC version 3.0 criteria

16.4.2 Secondary Endpoints

16.4.2.1 Disease-free survival is defined as the time from registration to the documentation of a first failure where a failure is the recurrence of breast cancer or a diagnosis of a second primary cancer.

16.4.2.2 Overall survival is defined as the time from registration to death due to any cause.

16.5 Analysis plan for each treatment schedule:

16.5.1 Immunologic Parameters

16.5.1.1 All eligible patients who have completed one cycle of treatment are evaluable of the analysis of the immunologic parameters.

16.5.1.2 For each of the immunologic parameters, a plot of the parameter level against time will be constructed such that each patient is represent by a line connecting that patient's data points. These plots will enable visual assessment of patterns of change and variability within a parameter as well as a visual assessment of whether the immunologic parameters peak or fall at similar time points.

16.5.1.3 Also, for each of the immunologic parameters, a plot of the percent change from pre-treatment levels against time will be constructed such that each patient is represent by a line connecting that patient's data points. These plots will enable visual assessment of time trends within a parameter controlling for pretreatment levels.

16.5.2 Adverse Events

16.5.2.1 All eligible patients who received at least one vaccination is evaluable for toxicity.

16.5.2.2 The frequency of those hematologic and non-hematologic toxicities considered at least possibly related to treatment will be tabulated by its severity.

16.5.2.3 Circumstances surrounding any treatment-related death will be reported.

16.6 As this is a pilot study, no formal hypothesis tests comparing treatment schedules are planned. An immunization strategy will be considered for further testing if at least 7 out of 10 patients treated with that strategy had a ≥2-fold increase in the percentage of vaccine-peptide specific CD8+ T cells during the course of treatment, with tolerable toxicity.

16.7 The principal investigator and study statistician will review the study every 3 months to identify potential accrual, toxicity, or endpoint problems. In addition,

this study will be monitored by the Cancer Center Data Safety Monitoring Board

16.8 Inclusion of Women and Minorities

This study will be available to all eligible patients, regardless of race or ethnic group. There is no information currently available regarding differential agent effects in subjects defined by gender, race, or ethnicity. The planned analyses will, as always, look for differences in treatment effect based on gender and racial groupings. The sample sizes of this pilot study, however, are not sufficient to provide power for such subset analyses.

To predict the characteristics of patients likely to enroll in this trial we have reviewed registration to (non-North American Breast Cancer Intergroup) NCCTG breast cancer clinical trials by race. This revealed that roughly 3% of patients registered into cancer trials during the past five years could be classified as minorities. This would suggest that only 1 or 2 patients in the study sample are expected to be classified as minorities. This precludes the possibility of a separate subset analysis beyond simple inspection of results for the 1 or 2 minority patients.

17.0 Pathology Considerations:

Central review of HER-2 and MUC1 staining.

18.0 Data Collection Procedures

18.1 Data will be entered into the computer within 2 weeks after each evaluation of the patient. After the patient goes off treatment, follow–up information will be collected and entered as specified in Section 18.2. If patient is still alive after 2 years have elapsed from on-study date, no further follow-up is required.

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Mucin 1-Specific Immunotherapy in a Mouse Model of Spontaneous Breast Cancer

*Pinku Mukherjee, *Cathy S. Madsen, *Amelia R. Ginardi, *Teresa L. Tinder, †Fred Jacobs, †Joanne Parker, †‡Babita Agrawal, †B. Michael Longenecker, and *Sandra J. Gendler

**Department of Biochemistry and Molecular Biology, Mayo Clinic Scottsdale, Scottsdale, Arizona, U.S.A.; †Biomira, Incorporated, Edmonton; ‡Current address: University of Alberta, Department of Surgery, Alberta, Canada.*

Summary: Human mucin 1 (MUC1) is an epithelial mucin glycoprotein that is overexpressed in 90% of all adenocarcinomas including breast, lung, pancreas, prostate, stomach, colon, and ovary. MUC1 is a target for immune intervention, because, in patients with solid adenocarcinomas, low-level cellular and humoral immune responses to MUC1 have been observed, which are not sufficiently strong to eradicate the growing tumor. The hypothesis for this study is that enhancing MUC1-specific immunity will result in antitumor immunity. To test this, the authors have developed a clinically relevant breast cancer model that demonstrates peripheral and central tolerance to MUC1 and develops spontaneous tumors of the mammary gland. In these mice, the authors tested a vaccine formulation comprised of liposomal-MUC1 lipopeptide and human recombinant interleukin-2. Results indicate that when compared with untreated mice, immunized mice develop T cells that express intracellular IFN-γ, are reactive with MHC class I H-2D^b/MUC1 tetramer, and are cytotoxic against MUC1-expressing tumor cells in vitro. The presence of MUC1-specific CTL did not translate into a clinical response as measured by time of tumor onset, tumor burden, and survival. The authors demonstrate that some of the immune-evasion mechanisms used by the tumor cells include downregulation of MHC-class I molecule, expression of TGF-β2, and decrease in IFN-γ -expressing effector T cells as tumors progress. Finally, utilizing an injectable breast cancer model, the authors show that targeting a single tumor antigen may not be an effective antitumor treatment, but that immunization with dendritic cells fed with whole tumor lysate is effective in breaking tolerance and protecting mice from subsequent tumor challenge. A physiologically relevant spontaneous breast cancer model has been developed to test improved immunotherapeutic approaches. **Key Words:** Immune evasion mechanisms—MUC1-specific cytotoxic T lymphocytes—Spontaneous mouse models of cancer—Tolerance—Transgenic mice.

Human cancers frequently express abnormal or altered self-proteins that are potentially immunogenic and trigger immune recognition. Low-level humoral and cellular immune responses to several antigens, including mucin 1 (MUC1), HER2/neu, p53, and PSA, are present in a significant proportion of patients with early and late stage cancer. It is important to determine how to obtain an effective immune response, since the native immune responses fail to eradicate tumors. In this study, human MUC1 is the tumor antigen of choice, as it is widely expressed on most solid adenocarcinomas. Although MUC1 is a self-molecule that is normally expressed on epithelial cells lining ducts and glands at low levels, it is a target for immunotherapy because it is significantly altered in expression during tumorigenesis. There is a

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Address correspondence and reprint requests to Sandra J. Gendler, Mayo Clinic Scottsdale, S.C. Johnson Medical Research Center, 13400 East Shea Boulevard, Scottsdale, AZ 85259, U.S.A.; e-mail: gendler.sandra@mayo.edu

large increase in the amount of MUC1 expressed on cells and in circulation. Its distribution is no longer restricted to the apical surface of the ducts and glands, but it is found throughout the tumor mass and on the surface of tumor cells. Most importantly, the glycosylation is altered. Oligosaccharides are shorter and fewer in number, revealing immunodominant peptide sequences that on normal cell surfaces would be sequestered by glycosylation. Underglycosylation of MUC1 reveals peptide epitopes recognized by cytotoxic T lymphocytes (CTL) that can kill tumor cells expressing this form of MUC1 (1–4).

The recent description of MUC1 as a target for CTL has raised interest in using this protein as a target for immunotherapy. In 2002, cancers that express MUC1 accounted for approximately 72% of new cases and for 66% of the deaths (5). These observations have prompted clinical vaccination trials aimed at boosting the weak immune responses to therapeutic levels. However, human clinical testing should ideally be preceded by extensive animal model studies to show that the concepts can be translated into efficacious therapy for cancer.

Until now, all MUC1-specific immunotherapy has been tested in mice using injected tumor cells (6–9). In this study, we have characterized the development of a mouse line that expresses human MUC1 as a self-molecule and spontaneously develops MUC1-expressing tumors of the mammary gland. We have developed this line by mating mice that carry the human MUC1 transgene driven by its own promoter (MUC1.Tg mice) (10) with oncogenic mice that carry the polyoma middle T antigen (MTag) driven by the MMTV promoter (11). The MUC1.Tg mice are not a model of MUC1 overexpression, rather the mice express human MUC1 in a developmentally regulated and tissue-specific fashion, as its own promoter drives the transgene. Mice transgenic for this protein develop B and T cell tolerance and are refractory to immunization with the protein encoded by the transgene (10). These double transgenic mice are designated MUC1-expressing mammary tumor (MMT) mice. We have analyzed MUC1 expression in MMT mice as tumor progresses in the mammary gland and characterized MUC1-specific native immune responses that develop during tumor progression. We further tested the efficacy of a MUC1-specific vaccine treatment in MMT mice and determined some of the immune-evasion mechanisms used by the tumor cells. Finally, using an injectable model, we demonstrate that targeting multiple tumor antigens rather than a single antigen may be more effective in eliciting an antitumor response. Breaking tolerance and eliciting a sustained antitumor immune response against the growing tumor are key to developing improved and novel immunotherapies for cancer. Our mouse model provides an improved system for evaluating the efficacy of antitumor vaccine formulations in vivo within the context of existing tolerance mechanisms.

MATERIALS AND METHODS

MUC1-Expressing Mammary Tumor Mouse Model

MUC1.Tg mice are bred with MTag oncogene-expressing mice. Double transgenic female mice spontaneously develop breast tumors and are designated as MMT. Male mice were discarded from this study. MUC1.Tg mice were developed in our laboratory (10). MTag mice were a kind gift from Dr. W.J. Muller (McMaster University, Hamilton, Ontario, Canada) (11). All mice are congenic on the C57/BL6 background at $N > 10$ (12). All mice were bred and maintained in specific pathogen-free conditions in the Mayo Clinic Scottsdale Natalie Schafer Transgenic Animal Facility. All experimental procedures were conducted according to IACUC guidelines.

PCR Screening

PCR was used to routinely identify MUC1 transgene and MTag oncogene to determine mice that were positive for both transgenes. PCR was carried out as previously described (10,13). The primer pair used for MUC1 transgene are 5′-CTTGCCAGCCATAGCACCAAG-3′ (bp. 745 to 765) and 5′-CTCCACGTCGTGGACATTGATG-3′ (bp. 1086 to 1065). Primer pairs for MTag transgene are 5′-AGTCACTGCTACTGCACCCAG-3′ (bp 282 to 302) and 5′-CTCTCCTCAGTTCCTCGCTCC-3′ (bp 817 to 837). The amplification program for MTag and MUC1 consisted of one 5-minute cycle at 95°C and 40 30-second cycles at 95°C, 1 minute at 61°C, and 30 seconds at 72°C followed by one 10-minute cycle at 72°C. The PCR product of each reaction was analyzed by size fractionation through a 1% agarose gel. Amplification of MUC1 resulted in approximately a 500 bp fragment and of MTag in a 480 bp fragment.

Cell Lines

Bl6 melanoma cells transfected with either full-length human MUC1 gene (Bl6.MUC1) or a neomycin resistance gene (Bl6.neo) were used as target cells for CTL assays. C57mg breast cancer cells transfected with full-length human MUC1 gene (C57mg.MUC1) were used as an injected model of breast cancer. Dr. Tony Hollingsworth from Eppley Cancer Center, University of Ne-

braska provided the B16.MUC1 cells, and our laboratory generated the C57mg.MUC1 cells. Bl6.MUC1 and B16.neo were maintained in DMEM media with 10% fetal bovine serum (FBS), penicillin (50 U/mL), and streptomycin (50 μg/mL) supplemented with 300 μg/mL G418. One day before conducting the ^{51}Cr-release assay, cells were treated with 5 ng/mL IFN-γ (Pharmingen, San Diego, CA, U.S.A.). Flow cytometry was routinely used to test cells for the presence of MUC1 and MHC class I. C57mg.MUC1 was maintained in DMEM with 10% FBS, penicillin (50 U/mL) and streptomycin (50 μg/mL), supplemented with 150 μg/mL G418 and 20 ng/mL insulin (Sigma, St. Louis, MO, U.S.A.).

Analysis of MMT

Tumors

From 8 weeks of age until sacrifice, control and immunized MMT mice were palpated once a week for presence of mammary tumors. Palpable tumors were measured by calipers, and tumor weight was calculated according to the formula: grams = (length in cm) × (width)^2/2 (14). In accordance with IACUC regulation, mice were killed when tumors reached 10% of body weight. Mice were carefully observed for signs of ill health, including lethargy, abdominal distention, failure to eat or drink, marked weight loss, and hunched posture.

Evaluation of Pulmonary Metastatic Lesions

The presence and number of pulmonary metastases was determined by gross morphologic assessment using low power microscopy.

Immunohistochemistry

Tumors were obtained from control and immunized MMT mice at time of sacrifice. To determine MUC1 levels in nonimmunized MMT mice as tumors progressed, we collected tumors at various time points during tumor progression. Tumors were fixed in methacarn followed by 70% ethanol, paraffin embedded, and step-sectioned for immunohistochemical analysis. MUC1 antibodies used were CT2, an Armenian hamster monoclonal antibody that recognizes mouse and human cytoplasmic tail (CT) region of MUC1 (15), and B27.29, a mouse monoclonal antibody with epitopes in the tandem repeat (TR) domain of MUC1 (16). B27.29 is specific for human MUC1 and does not cross react with mouse Muc1. The TR antibodies are glycosylation-sensitive in the mammary gland. Antibody to TGF-β2 was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, U.S.A.). Secondary antibody was a swine-antirabbit conjugated to HRP for TGF-β2 (Dako, Carpinteria, CA, U.S.A.); and a goat antihamster conjugated to HRP for CT2 (Jackson Labs., West Grove, PA, U.S.A.). HRP-conjugated B27.29 was obtained from Biomira, Inc. (Edmonton, Canada). Antibody staining was blocked with the appropriate peptides.

MUC1-Specific ELISA

Serum MUC1 levels were determined using the Truquant BR EIA supplied by Biomira Inc. (Edmonton, Canada) (17). A MUC1 catcher antibody was used to capture serum MUC1, which was detected using HRP-conjugated secondary antibody and an appropriate substrate.

Cytotoxic T Lymphocyte Assays

Determination of mature CTL activity was performed using a standard ^{51}Cr-release method with no in vitro peptide stimulation. Splenocytes from immunized and nonimmunized MMT mice were harvested by passing through a nylon mesh followed by lysis of red blood cells using Pharmlyze (0.45% ammonium chloride solution purchased from Pharmingen). These cells served as effectors. B16.MUC1 that expressed high levels of MUC1 as determined by flow cytometry analysis with B27.29 monoclonal antibody were used as target cells. For better presentation of MUC1 antigen, B16.MUC1 target cells were treated with 5 ng/mL IFN-γ 1 day before the assay to upregulate MHC class I surface expression. B16 melanoma cells transfected with vector that contains the neomycin resistance gene but no MUC1 (B16.neo) were used as control target cells. Specific ^{51}Cr-release was calculated according to the formula: (experimental CPM − spontaneous release CPM/maximum release CPM − spontaneous release CPM) × 100. Spontaneous release in all experiments was less than 15% of maximum release. To determine native immune response in nonimmunized MMT mice, we performed CTL assays at various time points during tumor progression (6, 9, 12, 15, 18, and 21 weeks). For these studies, splenocytes (1×10^6/mL) from nonimmunized MMT mice were stimulated for 6 days in vitro with 1×10^5/mL irradiated (3,000 Rads) bone marrow-derived dendritic cells (DC). Before irradiation, DC were pulsed with MUC1 TR peptide (24mer, TAPPAHGVTSAPDTRPAPGSTAPP) at 10 μg/mL for 1 day. Murine interleukin (IL)-2 (100 U/mL) (Pharmingen) was also added to the culture. At day 6 of culture, effector cells were used in the ^{51}Cr-release assay with

B16.MUC1 as target cells. In some experiments, B16.neo cells were used as control target cells.

Flow Cytometry

Single cells from peripheral blood lymphocytes (PBLs) collected from immunized and nonimmunized MMT mice at various times after immunization were analyzed by two-color flow cytometry for lymphocyte activation markers and intracellular cytokines. This included early and late surface activation markers, CD69 and CD25 (IL2-R) on $CD4^+$ and $CD8^+$ T cells. Intracellular cytokine levels were determined after cells were treated with brefeldin-A (Golgi-Stop; Pharmingen) according to the manufacturer's recommendation (4 μL/1.2 × 10^7 cells/6 mL for 3 hours at 37°C before staining). Cells were surface labeled with CD4, CD8, or CD11c antibodies before permeabilizing with the Pharmingen permeabilization kit (Cat #554722, containing 0.005% saponin and 4% formaldehyde) and staining for intracellular IFN-γ, IL-2, IL-4, IL-5, IL-10, or IL-12. Tumor cells from freshly dissociated MMT tumors were stained with fluorescently labeled antipan-cytokeratin and anti-MHC class I H-$2D^b$ and H-$2K^b$. All fluorescently labeled antibodies were purchased from Pharmingen except for antipan-cytokeratin, clone C-11, purchased from Sigma (St. Louis, MO, U.S.A.). Flow cytometric analysis was done on Beckton Dickinson FACscan using the Cell Quest program.

MUC1-Tetramer Staining

MHC class I H-$2D^b$ tetramers containing MUC1 TR peptide APGSTAPPA were created by Dr. Larry Pease at Mayo Clinic Rochester. PBLs were stained with 0.1 mg/mL H-$2D^b$/MUC1 tetramer (directly conjugated to phycoerythrin) for 1 hour on ice. To confirm specificity, an H-$2D^b$ tetramer containing an irrelevant peptide was used as control ($VP2_{121-130}$). The cells were then stained with CD8 and CD4 antibodies conjugated to FITC for 15 minutes before being analyzed by two-color flow cytometry.

Preparation of Dendritic Cell-Pulsed Liposomal MUC1 TR Lipopeptide

Dendritic cells were derived from C57BL/6 bone marrow cells according to the method described (18). Briefly, bone marrow cells were flushed from tibia, and femur and red blood cells were lysed with ammonium chloride. Cells (1 × 10^6/mL) were cultured in DMEM with 10% FBS, 1% glutamax, 50 U/mL penicillin, 50 μg/mL streptomycin (DMEM complete medium), supplemented with 10 ng/mL murine GM-CSF (Pharmingen) and 10 ng/mL murine IL-4 (Pharmingen). At day 7, nonadherent cells were removed, washed, resuspended (1 × 10^6/mL) in fresh DMEM complete media supplemented with 5 ng/mL GM-CSF. Adherent cells were re-fed fresh DMEM complete media supplemented with 5 ng/mL GM-CSF. Between days 10 and 13, we obtained approximately 70–75% cells that showed DC phenotype by flow cytometry. DC were fed with MUC1 TR lipopeptide that was enclosed in liposomes according to the method provided by Biomira, Inc. (19). Briefly, 2 mL of the liposomal MUC1 TR formulation was fed to 200 × 10^6 DC in a total volume of 20 mL, 1 day before injection. Mice were injected i.p. with 1 × 10^6 MUC1 TR pulsed DC per mouse in 200 μL volume. The first two immunizations at weeks 3 and 5 consisted of DC-fed liposomal MUC1 formulation. Thereafter, mice were immunized with liposomal MUC1 vaccine in PBS.

Vaccination Strategy

The vaccine formulation consisted of a lipid derivative of a 25mer MUC1 TR peptide (STAPPAHGVTSAPDTRPAPGSTAPP), which was incorporated into liposomes along with Lipid A as adjuvant. The vaccine was supplied as a sterile powder formulated at Biomira, Inc. by a proprietary method. Upon reconstitution with sterile saline for injection, it contained 400 μg/mL of MUC1 lipopeptide, 200 μg/ml of Lipid A (Avanti Polar Lipids, Inc., Alabaster, AL, U.S.A.) and 20 mg/mL of carrier lipids in multilamellar vesicles with a mean particle size of 2 to 3 μm. For the preparation of pulsed DC, particle size of the vaccine was reduced to < 200 nm by ultrasonication. Randomized preclinical trials were performed in MMT mice starting at 3 weeks of age.

Treatment Arms Include

Treatment arms include (a) liposomal MUC1 TR lipopeptide (L-MUC1-TR) (200 μg/mouse/250 μL s.c.) + L-IL-2 (20,000 U/mouse/100 μL i.p.), (b) L-MUC1-TR, (c) L-IL-2 (20), (d) empty liposomes, and (e) no treatment. We compared the immune responses that developed during treatment and used tumor onset, tumor burden, and survival as the endpoints for determining the clinical effectiveness of the vaccine. A schematic of the immunization protocol is shown in Figure 1.

Dendritic Cells Pulsed with C57mg.MUC1 Tumor Lysate

DCs were prepared as described above. Lysates from C57mg.MUC1 cells were made in tissue lysis buffer con-

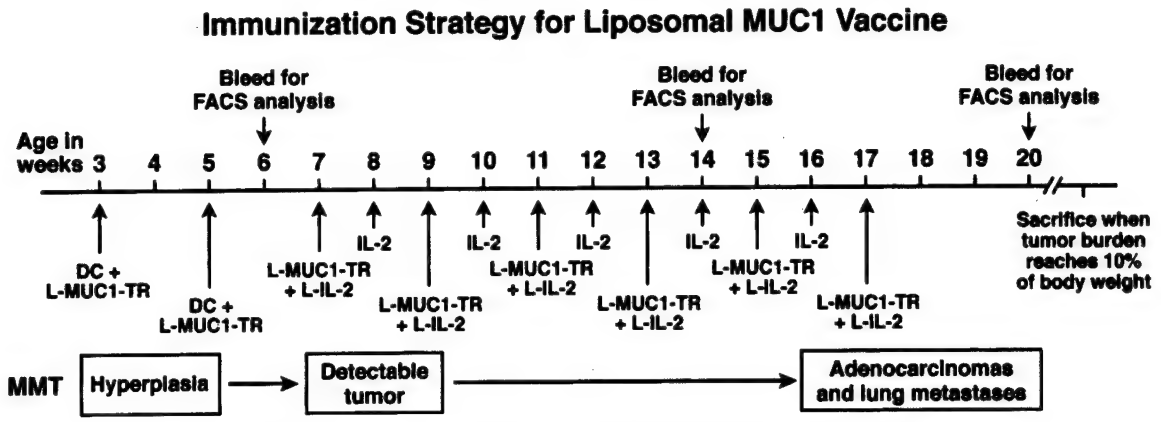

FIG. 1. Schematic representation of immunotherapy strategy in MMT mice.

taining 20 mmol/L Hepes, 0.15 mol/L NaCl, and 1% Triton X-100. Cells were sonicated using a Branson Sonifier 450 (VWR Scientific) at 20% duty cycle setting with three pulses of 10 seconds each. Immature 7-days-old DC were pulsed with 20 ng/mL of the tumor lysate for 1 day and then matured with 5 ng/mL TNF-α (Pharmingen) for 1 day. MUC1.Tg mice received two intradermal injections, 2 weeks apart of tumor lysate-pulsed DC at 1×10^7 DC per mouse. One week after the last injection, mice were challenged with 2×10^6 C57mg.MUC1 tumor cells (subcutaneously).

Statistical Analysis

p values are from the one-way ANOVA *F* test for comparing the treatment groups simultaneously and from the pairwise contrasts from a one-way ANOVA model. p values for lung metastasis are from the Pearson χ^2 statistic and from Kruskal-Wallis test.

RESULTS

MMT Mice Develop Spontaneous Mammary Gland Cancer and Lung Metastases

MUC1.Tg mice were bred with mice carrying the MMTV-driven polyoma middle T antigen (MT) to create MMT mice. Prior to generating the double transgenics, the MTag mice had been backcrossed 10 generations onto C57BL/6 mice, making them congenic (12). In the MMT mice, mammary gland tumors are induced by the action of a potent tyrosine kinase activity associated with

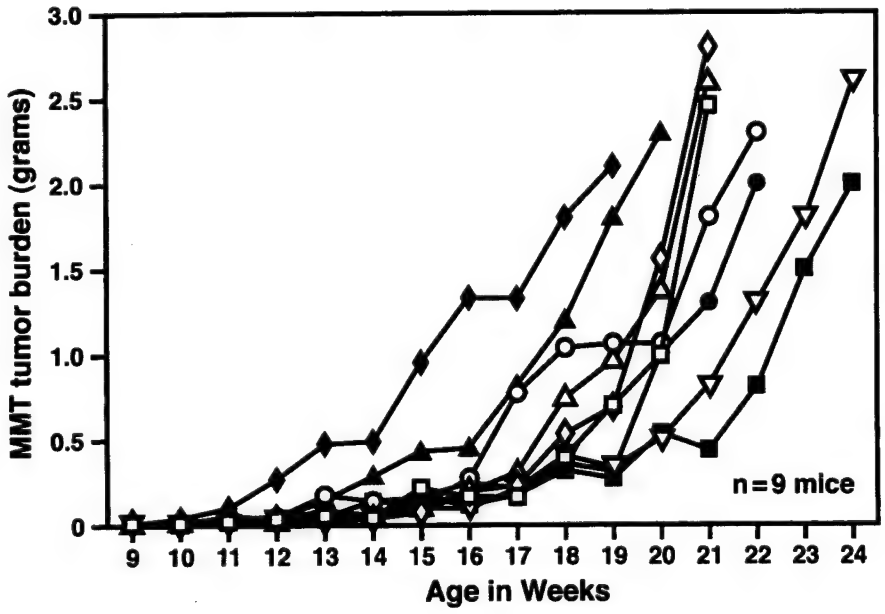

FIG. 2. Tumor growth curve in MMT mice as a function of age. Tumor burden was determined by palpation beginning at 8–9 weeks of age and calculated by the formula: gm = (Length × Width^2) × 0.5. Most mice develop palpable tumors by 10–11 weeks. Tumors reach 10% of mouse body weight between 20 and 24 weeks at which point mice are killed. Data for individual mice are shown (n = 9 mice).

the polyoma virus middle T antigen driven by the mouse mammary tumor virus long terminal repeat (MMTV) (11). Middle T specifically associates with and activates the tyrosine kinase activity of a number of c-src family members, eliciting tumors when a threshold level of gene product has been attained. This promoter is transcriptionally active throughout all stages of mammary gland development and results in widespread transformation of the mammary epithelium and the rapid production of multifocal mammary adenocarcinomas. Hyperplastic alveolar nodules (HANs) can be detected by whole mount as early as 21 days and palpable mammary gland tumors are detectable from approximately 49 days onwards. Tumor progression is quite rapid, reaching 10% of body weight by approximately 20 to 24 weeks (Fig. 2). 100% of the female MMT mice get tumors. Tumors arise with synchronous kinetics and are highly fibrotic with dense connective tissue separating individual nests of tumor cells, a pathology that closely resembles scirrhous carcinomas of the human breast (21). These mice exhibit metastasis in the lungs (30 of 49 MMT mice tested or 60%) and micro-metastasis in the bone marrow by 4 months of age. After the observation that epithelial cells were present in several of the 20- to 24-week-old MMT-derived bone marrow cultures, we examined if bone marrow micro-metastasis was occurring in these mice. Bone marrow metastasis was determined by staining bone marrow cells from MUC1.Tg mice and MMT mice with pan-cytokeratin and MUC1, markers commonly used to detect epithelial tumor cells. Cells positive for both markers were determined by two-color flow cytometry as well as by confocal microscopy and represent micro-metastasis (Figs. 3A and B). By flow cytometry, 3 to 4% of bone marrow cells were positive for both pan-cytokeratin and MUC1. It is important to note that mortality in patients with breast cancer is directly associated with lung and bone metastasis. The MMT mouse appears to be an appropriate model for human cancer and allows us to study the effects of self-tolerance, immunity, and autoimmunity to MUC1 as mammary tumors develop spontaneously.

Tumor Burden and Lung Metastasis in Immunized MMT Mice

We immunized mice with L-MUC1-TR ± L-IL-2. The first two immunizations administered to young (3- and 5-week-old) MMT mice used syngeneic DC loaded with

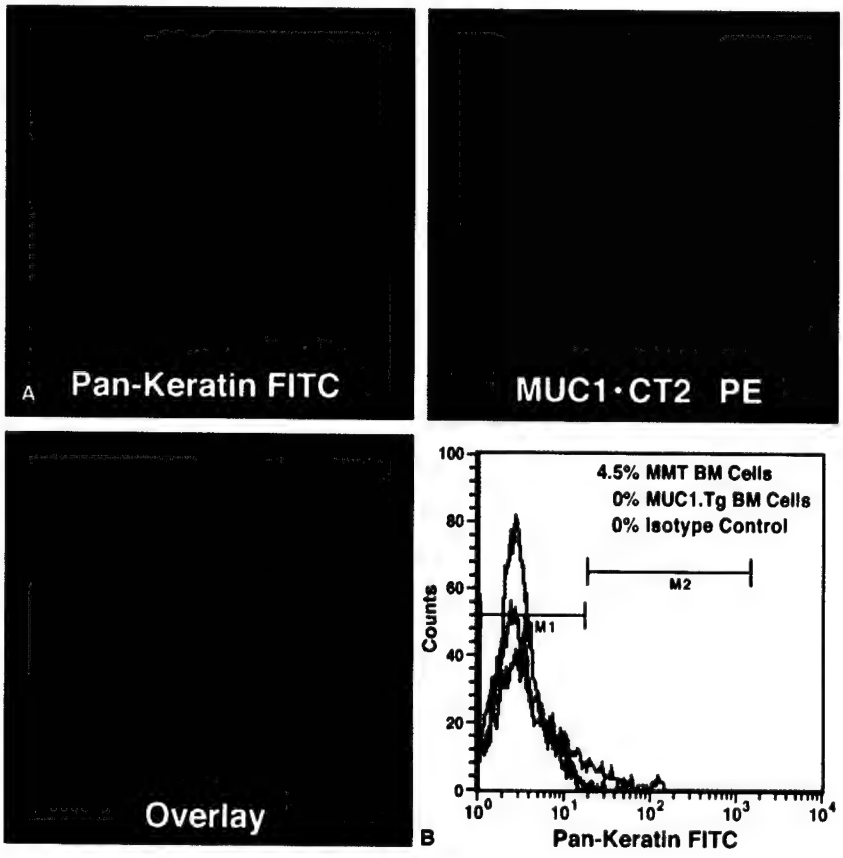

FIG. 3. Bone marrow metastasis is detected in MMT mice. **(A)** Confocal microscopy showing bone marrow cells from MMT mice positive for pan-cytokeratin (green) and MUC1 (red). Colocalization is shown in yellow. For MUC1 staining, a hamster monoclonal antibody, CT2 is used. **(B)** Representative histogram for two-color flow cytometric analysis of bone marrow cells derived from 22-week-old MMT mouse. The histogram represents percent cells stained positive for pan-cytokeratin that were gated on MUC1$^+$ cells. Two controls include bone marrow cells from age matched MUC1.Tg mice and isotype IgG.

liposomal MUC1 to induce strong immunity as young mice respond well to an antigen when presented in context of DC without the induction of tolerance (22). From week 7 onwards, mice were given L-MUC1-TR reconstituted in PBS (s.c.) and subsequently boosted with the same formulation every 2 weeks. L-IL-2 was administered (i.p.) every 2 weeks to the appropriate groups. Although no significant differences were observed in the onset and progression of the MMT tumors with immunization (Fig. 4A), by 18 weeks of age there was significant decrease ($p < 0.05$ to $p < 0.005$) in tumor burden between immunized and control MMT mice (Fig. 4B). To our surprise, empty liposomes elicited a similar response suggesting that the lipid A in the formulation may have an effect on the tumor. However, by 20 to 24 weeks, no significant differences in tumor burden between immunized and control mice were observed (Fig. 4A). Interestingly, we observed that treatment with L-IL-2 alone had significantly lower numbers of lung metastasis than mice in all other treatment groups and in untreated control mice (Fig. 4C). Incidence of metastasis was also lower in mice treated with L-IL-2 as compared with other treatment groups and untreated MMT mice (44% versus 64% in untreated MMT) (Table 1). These results did not reach significance due to low animal numbers. All mice were killed when tumors reached 10% of body weight, and, therefore, difference in survival was not observed. Because MUC1 is expressed on normal cells, we routinely examined mice for signs of autoimmunity after immunization. We monitored weight loss, food intake, general health, hunched back, and histopathology of various MUC1-expressing organs. No signs of autoimmunity were evident in our study group (data not shown).

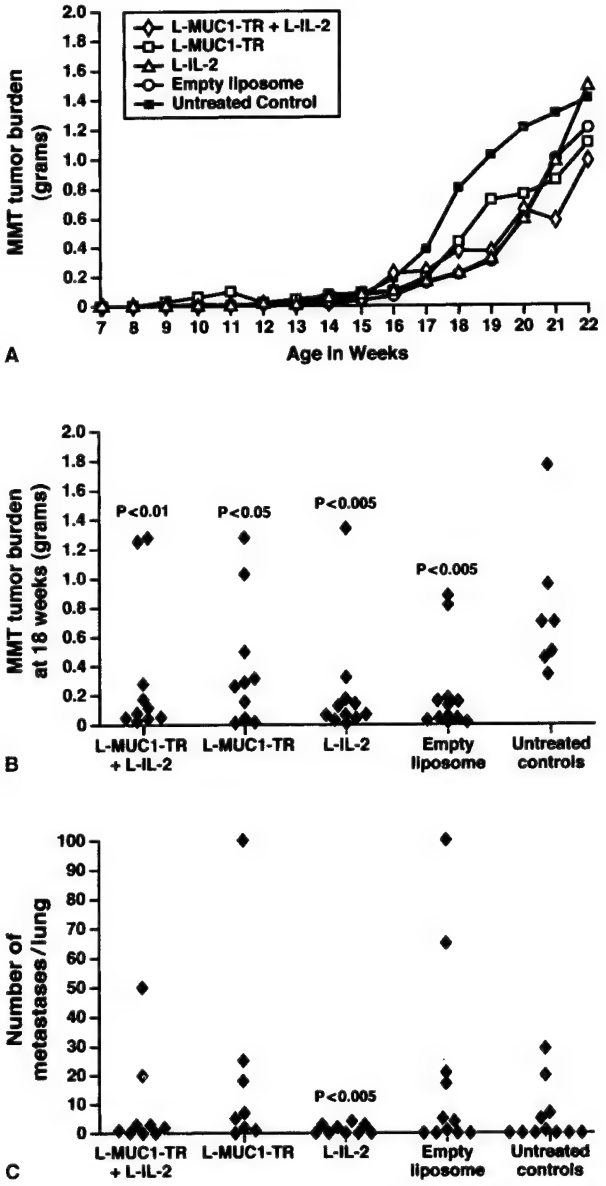

FIG. 4. **(A)** MUC1-specific immunization did not alter tumor burden in MMT mice. Tumor growth of immunized MMT mice versus untreated MMT mice. Tumor burden was determined by palpation beginning at 6–8 weeks and calculated by the formula: gm = (Length × Width^2) × 0.5. No significant difference was observed in mice immunized with MUC1 TR + L-IL-2 as compared with untreated control mice (n = 6–9 mice/group). **(B)** Tumor burden was lower in immunized MMT mice at 18 weeks of age. In treatment groups, tumor burden at 18 weeks was significantly lower as compared with untreated control mice. Individual mouse data are shown. p values are derived from the pairwise contrasts from a one-way ANOVA model. **(C)** L-IL-2 treatment reduces lung metastasis in MMT mice. The presence of pulmonary metastases was determined by counting gross morphologic disease using low power microscopy. Number of metastatic lesions counted in the lungs was significantly lower in mice treated with L-IL-2 as compared with other treatment groups and untreated control mice. Individual mouse data are shown (n = 8 to 11 mice/group). p values were from the Pearson χ^2 statistic and from Kruskal-Wallis test.

MUC1 Expression in MMT Tumors

Tumor sections from 6-, 12-, and 21-week-old nonimmunized MMT mice show strong heterogeneous expression of MUC1 as tumors progress (Fig. 5). Paraffin embedded tumor sections from 6-, 12-, and 21-week-old MMT mice were stained with two antibodies, one recognizing the CT of MUC1 (CT2) and the other recognizing the TR epitope of MUC1 (B27.29). CT2 antibody recognizes mouse and human CT of MUC1. It is a monoclonal antibody (Mab) raised in Armenian hamster and thus gives very little background when used to stain tissues from mice. Mammary tumors showed moderate expression of human MUC1, using Mab. B27.29, which reacts only with the human MUC1 and not with mouse

TABLE 1. *Percentage of MMT mice with lung metastasis*

Treatment	Number	%
L-MUC1 TR + L-IL-2	6/9	66
L-MUC1 TR	7/8	87
L-IL-2	4/9	44
No treatment	8/12	64

Pulmonary metastases was determined by counting gross morphologic disease using low power microscopy. The authors determined how many mice developed lung metastasis.

Muc1 (23). MUC1 was expressed throughout the cytoplasm and around the cells in a pattern similar to that observed in human breast carcinomas. Western blots of tumor lysates using B27.29 for detection showed protein expression (data not shown). Thus, mammary gland tumors that occurred spontaneously in the MMT mice expressed the transgene protein MUC1 in a manner similar to humans. Any alteration in MUC1 expression will be important in the immunologic recognition and presentation of the antigen. Immunization did not alter the MUC1 expression in the MMT tumors (data not shown).

Native Immune Responses Developing in MMT Mice as Tumors Progress

We have determined the native cellular and humoral immune responses in the nonimmunized MMT mice as tumors progressed. MMT mice were bled at 6, 14, and 21 weeks of age and PBLs were examined for T cells expressing early and late activation markers (CD69 and CD25), DC ($CD11c^+$ cells) expressing B7 costimulatory receptors, T cells and DC expressing intracellular cytokines such as IFN-γ, IL-2, IL-4, IL-5, IL-10, and IL-12. We also examined PBLs for presence of T cells recognizing $H\text{-}2D^b$/MUC1 tetramer. The results are tabulated in Table 2. Increases were observed in some of the markers (IL-2R and intracellular IL-4 and IL-12) at the 14-week time point but the difference was not statistically significantly and was no longer observed at 21 weeks. $CD11c^+$ and $B7^+$ cells were significantly lower at 21 weeks when the tumor burden was high as compared with 6 and 14 weeks, which may indicate a defect in the antigen presenting cells during tumor progression. Thus our data clearly show that no significant alterations in

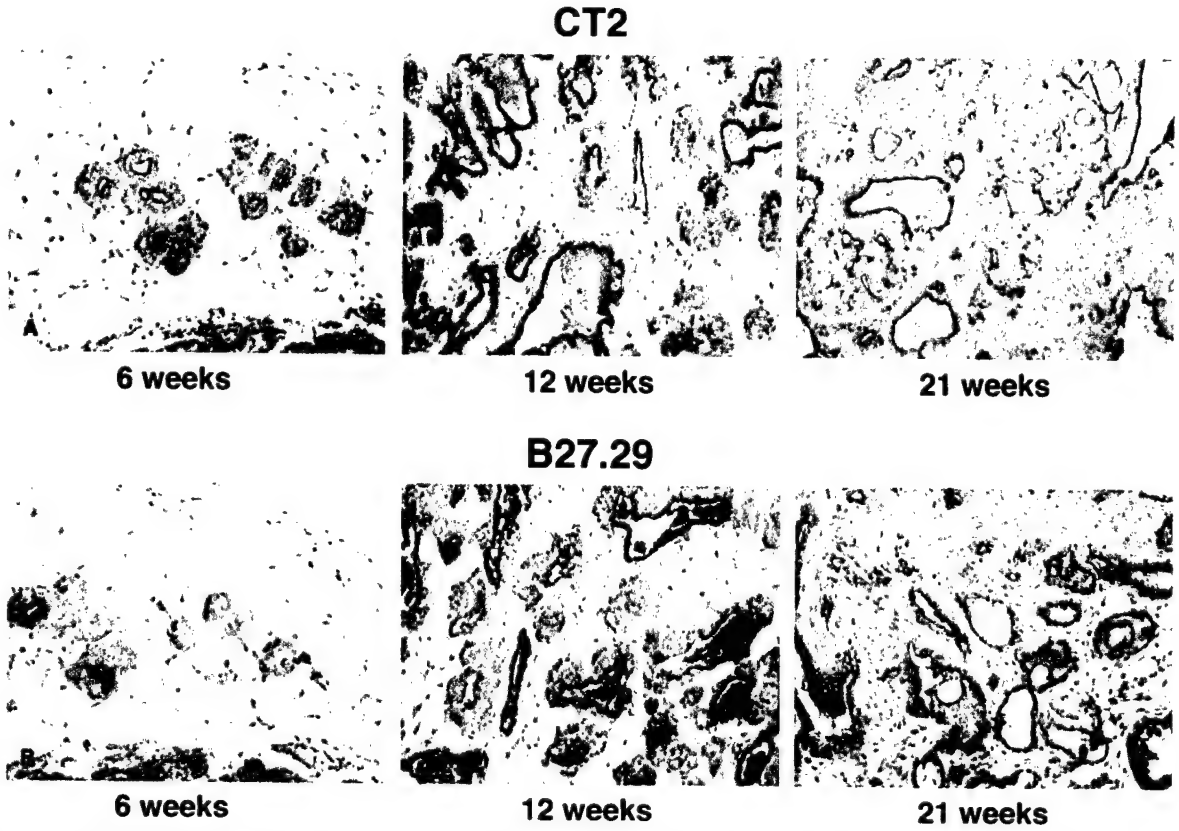

FIG. 5. MUC1 expression increases as tumors develop in MMT mice. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6-, 12-, and 21-week-old MMT mice were stained with **(A)** CT2, reactive with MUC1 cytoplasmic tail and **(B)** B27.29, reactive with MUC1 TR. Specific staining was observed on lumenal surface of mammary epithelial cells and staining pattern is similar to that seen in humans. Staining with these antibodies showed increase in MUC1 expression as tumors developed (original magnification ×200).

TABLE 2. *Immunophenotype of lymphocytes as tumors progress in MMT mice*

Markers	6-week MMT	14-week MMT	21-week MMT
IL-2R	1.58	3.28	0.28
CD69	0.75	0.30	0.17
IL-2	1.03	1.29	0.20
IFN-γ	0.50	0.73	0.38
IL-4	0.56	7.67	2.31
IL-5	0.16	2.61	2.28
IL-10	0.24	0.43	0.42
IL-12	0.26	6.43	2.69
B7	4.50	7.70	1.30*
CD11c	11.20	12.30	5.50*
MUC1-Tetramer	0.31	1.32	0.19

We have determined expression of early and late activation markers (CD69 and IL-2R) on T cells, as well as levels of intracellular cytokines produced by T cells (IFN-γ, IL-2, IL-4, IL-5, and IL-10) and dendritic cell (IL-12). The numbers represent percent positive cells stained with the specific antibody and analyzed by flow cytometry. Increases were observed in some of the markers (IL-2R and intracellular IL-4 and IL-12) at the 14-week time point that declined by 21 weeks but the difference was not statistically significantly. $CD11c^+$ and $B7^+$ cells were significantly lower at 21 weeks (when the tumor burden was high) as compared with 6 and 14 weeks. Average of n = 6 mice/group is shown. P values are from the pair-wise contrasts from a one-way ANOVA model.

*$p < 0.05$.

Th1 or Th2 immune phenotype occurs in MMT mice as tumors progressed. Presence of naturally occurring precursor CTL activity against MUC1 in 6-, 9-, 12-, 15-, 18-, and 21-week-old MMT mice was also tested and the data are presented in Figure 6. We were unable to detect precursor CTL activity in splenocytes at any time during tumor progression. With regards to humoral immune response, circulating antibody to MUC1 was undetectable by specific ELISA at any time during tumor progression (data not shown). These data taken together clearly indicate that naturally occurring cellular or humoral immune responses in nonimmunized MMT mice were nondetectable, which is in sharp contrast to the results reported previously for a similar model of pancreas cancer (3).

T Cell Immune Response in Immunized MMT Mice

Immunization elicited mature MUC1-specific CTL that were cytotoxic against B16.MUC1 tumor cells in vitro (Fig. 6). To determine MUC1 specificity, B16 cells transfected with vector alone (B16.neo) were routinely used as control tumor target and lysis of < 5% was observed making the CTL specific for MUC1 (data not shown). Both MHC-restricted and nonrestricted MUC1-specific CTL have been reported in the literature for human cancer (1,24); however, in MMT mice, we only detect restricted CTL. It is possible that these mice do possess unrestricted CTL but these CTLs may be difficult to detect in vitro and therefore have never been

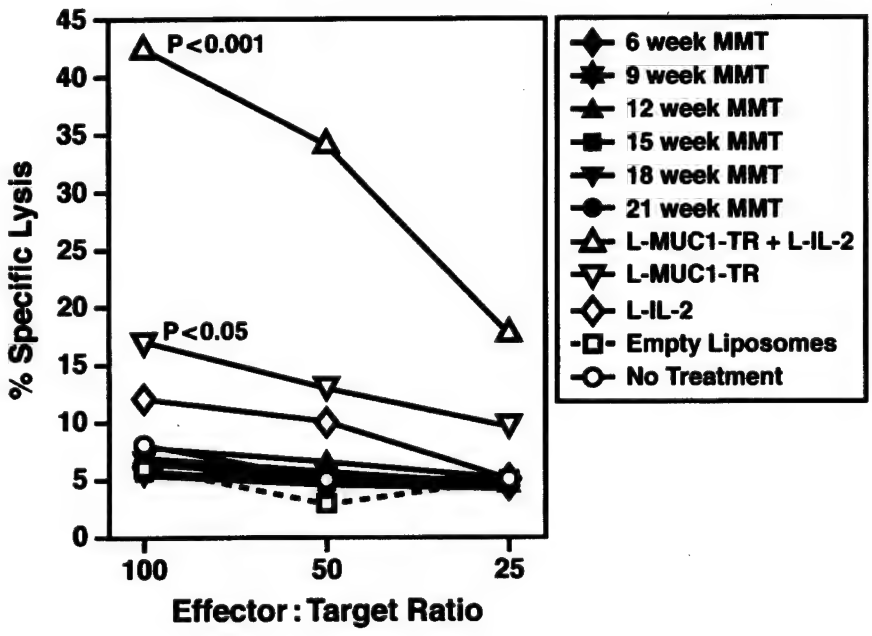

FIG. 6. Immunized MMT mice develop MUC1-specific CTLs. CTL activity in 6-, 9-, 12-, 15-, 18-, and 21-week-old untreated MMT mice was determined by a standardized 8-hour ^{51}Cr-release assay using B16 melanoma cells transfected with human MUC1 as target cells. Splenic T cells from MMT mice were used as effector cells. In immunized mice, CTL activity was determined at time of sacrifice when tumors reached 10% of body weight. The effector cells were not stimulated in vitro with MUC1-TR or cytokines. In untreated mice, no CTL activity was detected at any age. MMT mice in all treatment groups showed increase in MUC1-specific CTL with most lysis detected in mice immunized with L-MUC1-TR + L-IL-2. (*$p < 0.001$) as compared with untreated control. L-MUC1-TR immunization alone gave significantly higher CTL activity as compared with empty liposome group and untreated controls ($p < 0.05$). Specific lysis was calculated according to the formula: (experimental CPM − spontaneous CPM/maximum CPM − spontaneous CPM) × 100. Average of n = 6 mice/group is shown. p values are from the pairwise contrasts from a one-way ANOVA model. *Indicates comparison of untreated controls versus treatment groups.

reported previously in mice (25–28). Although the CTLs were cytotoxic in vitro, they had no effect on the growing tumor cells in vivo in MMT mice. This was determined by immunohistochemistry of MMT tumor sections in which MUC1-expressing tumor cells remained unchanged with immunization, suggesting that the MUC1-specific CTLs were not cytotoxic against the tumor cells in vivo (data not shown). To evaluate the T cell responses during immunization and as the tumors progressed, immunized and nonimmunized MMT mice were tail-bled at 6, 14, and 20 weeks, PBLs were isolated and tested for presence of a) T cells expressing intracellular IFN-γ and b) T cells recognizing H-2D^b/MUC1 tetramer. All treatment groups in immunized MMT mice showed increased numbers of T cells expressing intracellular IFN-γ by 10 to 14 weeks of age as compared with untreated control mice (Fig. 7A, p values shown in the figures). By 20 weeks of age, there was a decrease in these cell numbers suggesting that repeated immunization with MUC1 TR peptide or high tumor burden may affect T cell effector function. This observation correlates well with the decrease in tumor burden at 18 weeks in immunized mice, which does not last at 21 weeks (Fig. 4B). Similarly, T cells reactive with H-2D^b/MUC1 tetramer increased by 14 weeks in immunized MMT mice but no further increase was observed at 20 weeks (Fig. 7B), p values shown in the figures). Although some increase in percent T cells recognizing MUC1-tetramer was observed in the empty liposome treated mice versus untreated controls, significantly higher percent was observed in mice treated with L-MUC1-TR alone or with L-MUC1-TR + L-IL-2 ($p < 0.01$ and $p < 0.05$, respectively) versus empty liposome group. The increase in empty liposome group suggests that the lipid A present in the liposomal vaccine formulation may be sufficiently immunogenic in eliciting an anti-MUC1 response, albeit

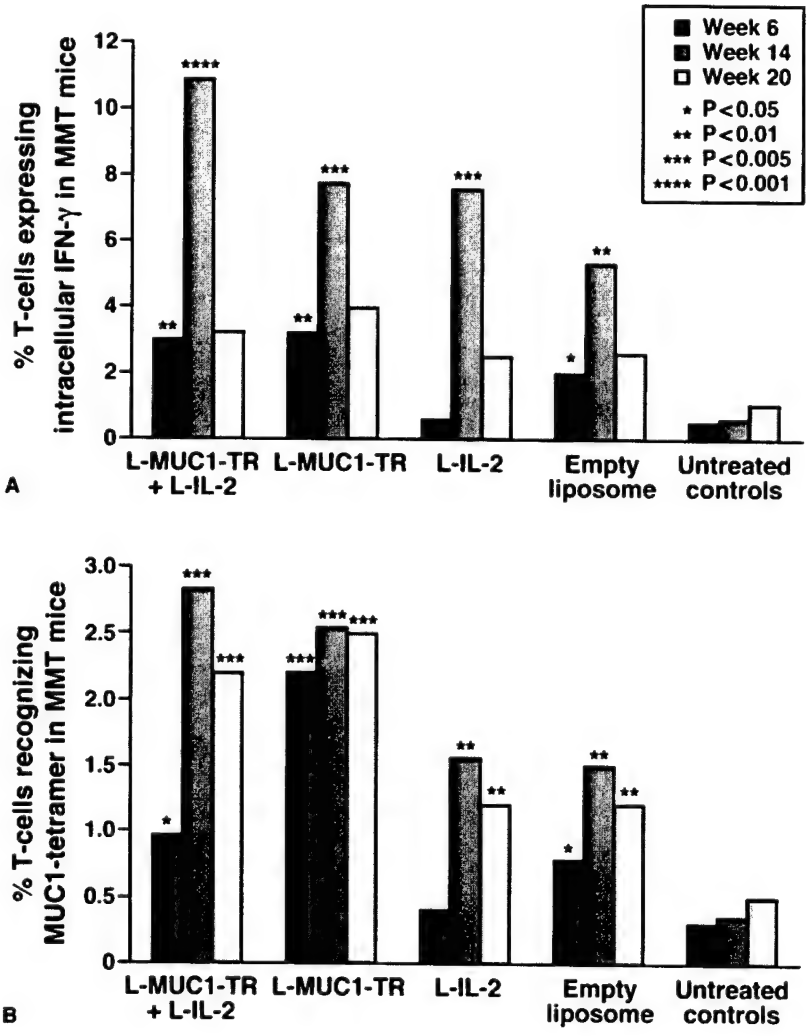

FIG. 7. MUC1-specific immunization elicits **(A)** T cells expressing IFN-γ and **(B)** T cells that recognize H-2D^b/MUC1 tetramer. At 6, 14, and 20 weeks after immunization, PBLs were analyzed for presence of T cells expressing IFN-γ and T cells recognizing H-2D^b/MUC1 tetramer. **(A)** Six weeks after immunization, mice in all treatment groups showed an increased percent of T cells expressing IFN-γ (**$p < 0.01$ for L-MUC1-TR groups). No difference in L-IL-2 treated group was observed at 6 weeks, as L-IL-2 treatment only begins at week 7. By 14 weeks after immunization, these T cells further increased. By 20 weeks after immunization, these cells decreased and were at levels similar to that observed at 6 weeks after immunization. At 14 weeks, percent T cells expressing intracellular IFN-γ was significantly higher ($p < 0.06$) in the L-MUC1-TR treated and L-IL-2 treated groups as compared with mice treated with empty liposome. Much higher significance ($p < 0.005$) was observed between L-MUC1-TR + L-IL-2 treated group versus empty liposome group. *Indicates comparison of untreated controls versus treatment groups. **(B)** Compared with untreated controls, mice in all treatment groups showed significant increase in percent T cells recognizing H-2D^b/MUC1 tetramer as early as 6 weeks after immunization. By 14–20 weeks after immunization, treated mice showed higher percent of circulating T cells recognizing H-2D^b/MUC1 tetramer. At all time points, percent T cells recognizing MUC1-tetramer was significantly higher ($p < 0.01$) in the L-MUC1-TR treated group as compared with mice treated with empty liposome. Significant difference ($p < 0.01$) was also observed between the L-MUC1-TR + L-IL-2 treated group versus empty liposome group at 14- and 20-week time points but not at the 6-week time point. No significant difference was observed between L-IL-2 treated group versus empty liposome group. *Indicates comparison of untreated controls versus treatment groups. Average of n = 6 mice/group is shown. p values are from the pairwise contrasts from a one-way ANOVA model.

not as strong as the vaccine formulation containing MUC1. Furthermore, the response seen with the empty liposome group did not translate into MUC1-specific CTL response (Fig. 6) nor did it correlate with elevated serum MUC1 levels in Figure 8A.

MUC1 Serum Levels in Immunized MMT Mice

As tumors progressed in the MMT mice, MUC1 serum levels increased only slightly as compared with age matched MUC1.Tg mice with maximum reaching to 1,500–2,500 U/mL of serum at 20 weeks of age. In age matched female MUC1.Tg mice, serum MUC1 levels ranged from 500 to 1,200 U/mL, which is likely to depend upon their estrous cycle status (data not shown). The low levels of circulating MUC1 may explain the lack of an immune response to MUC1 in nonimmunized MMT mice (Fig. 6 and Table 2). Immunization, however, significantly increased the serum MUC1 levels compared with untreated MMT mice (Fig. 8A), which corresponded directly to the increased CTL activity in these mice. These results suggested that high levels of circulating tumor antigen, MUC1, may activate MUC1-specific CTL that are capable of specifically lysing MUC1-expressing tumor cells in vitro (Fig. 6). We also detected low levels of circulating antibodies to MUC1 in

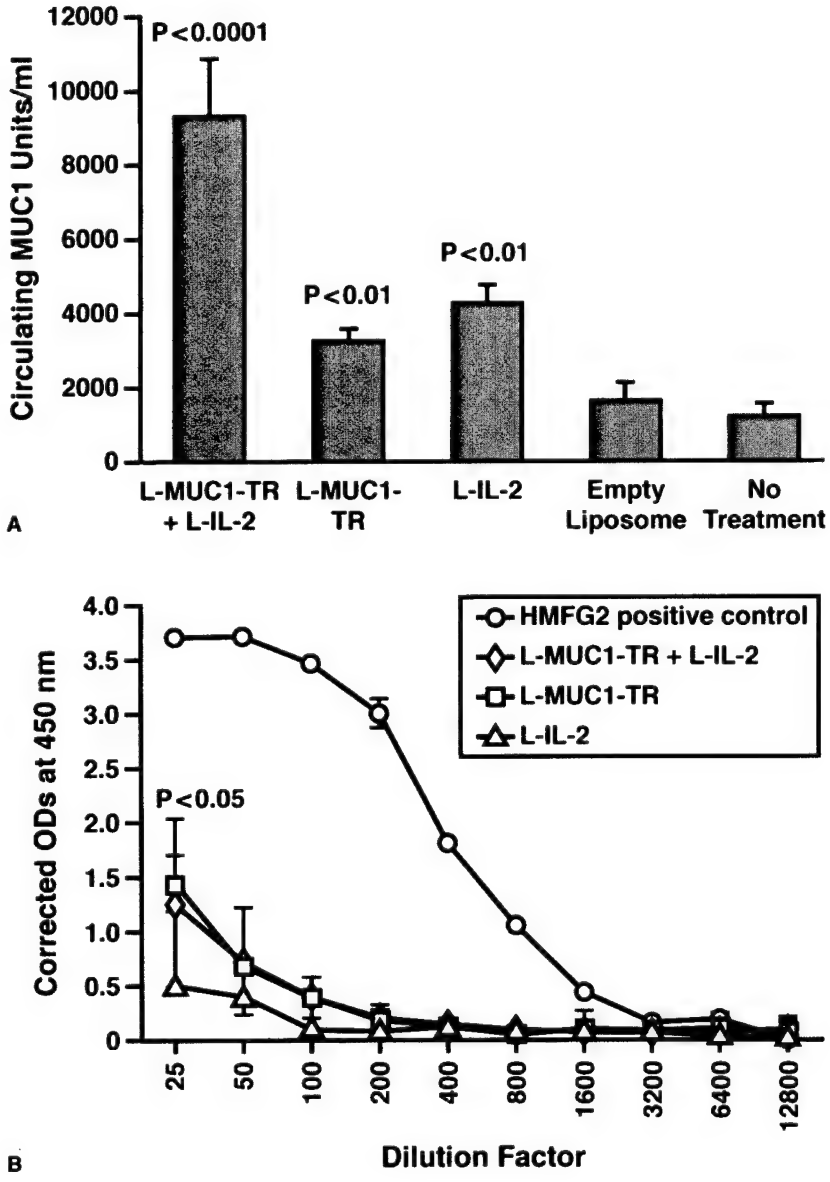

FIG. 8. Circulating MUC1 and anti-MUC1 levels in serum of immunized MMT mice. **(A)** Circulating MUC1 levels increase significantly with MUC1-specific immunization as well as with IL-2 treatment. **(B)** Low levels of MUC1 antibodies were detected in MMT mice immunized with MUC1 TR containing vaccine formulation. IL-2 treatment did not elicit MUC1-specific antibodies. Average of n = 6 mice/group is shown. p values are from the pairwise contrasts from a one-way ANOVA model. *Indicates comparison of untreated controls versus treatment groups.

the L-MUC1-TR + IL-2 treated mice (Fig. 8B) suggesting that immunization and the high level of circulating tumor associated MUC1 has changed the antigenic profile and elicited a low level humoral response to MUC1. Antibodies reactive with MUC1 have been reported in a small number of humans with breast cancer (29,30). Although a humoral response is often dismissed as being ineffective against solid tumors, it is still important that the response in the MMT mice once again parallels that in humans.

MHC Class I Expression in MMT Mice as Tumors Progress

Since the MUC1-specific CTLs have minimal effect in vivo, we postulated that the growing MMT tumor cells evaded immune recognition. We evaluated one of the well-characterized mechanisms by which tumor cells evade CTL killing, downregulation of their surface MHC class I molecules. We observed by two-color flow cytometry that percent cells positive for MHC class I and pan-cytokeratin are approximately 13% in 6-week-old MMT tumor mice which steadily decreases to <2% in 18-week-old tumor mice (Table 3) and that immunization was unable to upregulate these levels.

MMT Tumors Express TGF-ß

Another effective way a tumor cell evades T cell killing is to render the effector T cells nonfunctional by releasing immunosuppressive factors. One such factor is TGF-β that is capable of hindering T cell signaling and down regulating their function. We tested whether mammary tumors from MMT mice express TGF-ß by specific immuno-histochemical staining and demonstrate that MMT tumors express TGF-ß as early as 6 weeks of age and that the expression increases as the tumors progress (Fig. 9). MUC1-specific immunizations did not alter the expression of TGF-ß, suggesting that the tumors may be utilizing this mechanism to downregulate T cell activity in vivo and escape immune intervention. This phenomenon is also evident from the decrease in the numbers of IFN-γ expressing T cells (Fig. 7A) as tumor burden increases.

TABLE 3. *MHC class I expression on tumor cells*

Tumor age (wk)	MHC I expression
6	12.5 ± 2.0
12	4.1 ± 0.89
18	1.5 ± 0.5

Surface expression of MHC class I molecule on tumor cells decreases as tumors progress in MMT mice. FACs analysis demonstrating percent cells positive for pancytokeratin and MHC class I molecule ($H\text{-}2D^b/K^b$). n = 5 MMT mice.

Tumor Lysate Pulsed DC were Effective in Breaking Tolerance and Preventing Tumor Formation in MUC1.Tg Mice

Another plausible explanation for the failure of MUC1-specific immunization to eradicate MMT tumors is the utilization of a single tumor antigen as immune target. Recent findings suggest that tumor lysate-fed DC generate tumor-specific proliferative cytokine release and cytolytic reactivities in vitro as well as effectively prime mice to reject subsequent lethal challenges with viable parental tumor cells (31,32). Using an injectable tumor model, we show that MUC1.Tg mice immunized with DC pulsed with lysates prepared from C57mg.MUC1 cells were completely protected from subsequent challenge with C57mg.MUC1 tumor cells (Fig. 10). These results are promising, as we were able to break tolerance in the MUC1.Tg mice and protect them against tumor challenge. However, the challenge is to achieve these results in our spontaneous model, which is physiologically more appropriate and, to a large extent, mimics the human situation.

DISCUSSION

We describe a mouse model of spontaneous breast cancer that appropriately mimics human cancer and is an excellent model for testing novel immunotherapeutic strategies. Some important features of this model are the reproducible development of spontaneous mammary gland tumors, the occurrence of invasion and metastasis, the presence of an intact immune system, and the presence of a targetable and stable tumor antigen, MUC1. Most importantly, the tumor models resemble human cancer with regards to progression through various development stages of cancer and sensitivity to hormonal and stromal alterations. A recent study has established that MTag-induced hyperplasias, like early proliferative lesions seen in human breast, are heterogeneous with respect to their malignant potential. The study further establishes that MTag gene expression alone is insufficient to induce tumors and that additional events are required for tumorigenesis and metastasis (33).

In the MMT mice, as in humans, the mammary gland develops after birth, tumors are multifocal, grow rapidly, and are histologically homogeneous, highly fibrotic with dense connective tissue separating individual nests of cells. Tumors occur in a reasonable time frame to allow

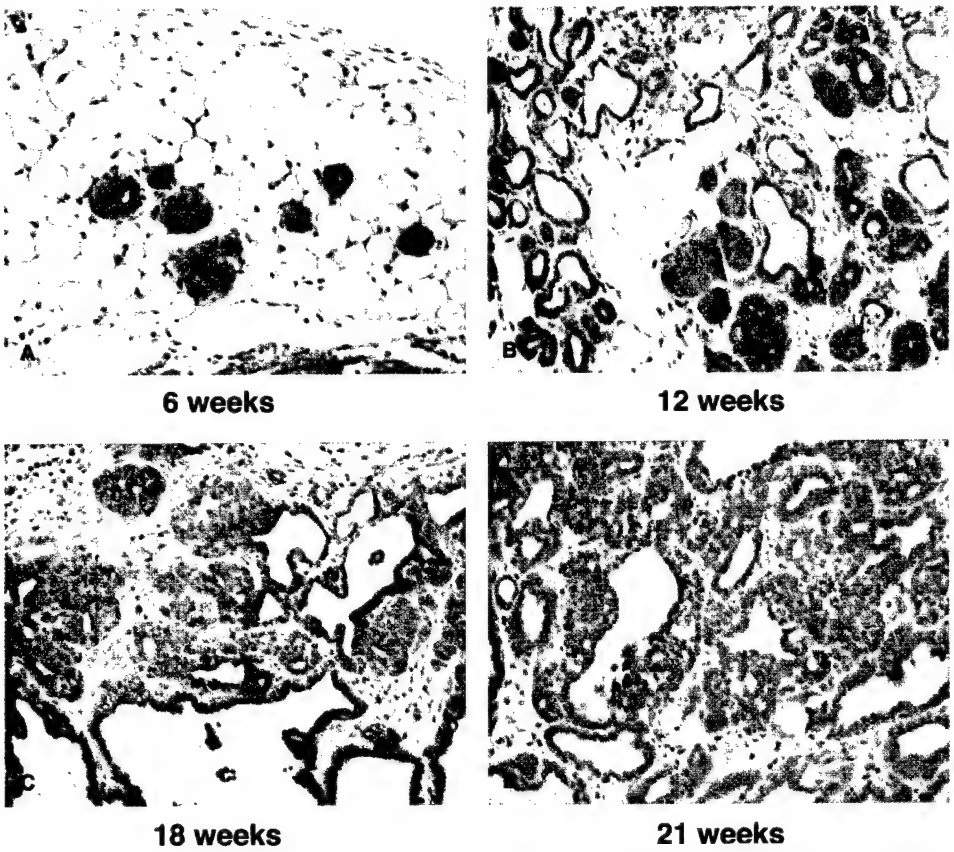

FIG. 9. Tumors from MMT mice express TGF-β as early as 6 weeks of age, which increases with time. Methacarn fixed and paraffin-embedded sections of mammary gland tumors from 6-, 12-, 18-, and 21-week-old MMT mice were stained with antibody to TGF-β2. MMT tumor at **(A)** 6 weeks, **(B)** 12 weeks, **(C)** 18 weeks, and **(D)** 21 weeks of age (original magnification ×200).

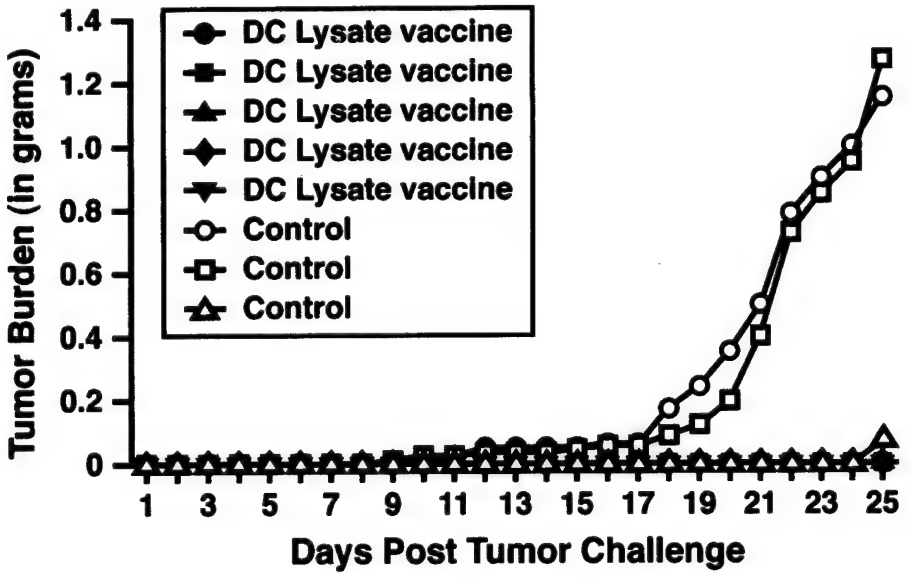

FIG. 10. Tumor progression in MUC1.Tg mice immunized with tumor lysate-pulsed dendritic cells (DC). MUC1.Tg mice received two intradermal injections, 2 weeks apart, of C57mg.MUC1 tumor lysate-pulsed DC at 1×10^7 DC per mouse. One week after the last injection, mice were challenged with 2×10^6 C57mg.MUC1 tumor cells (subcutaneously). All five immunized mice were protected from tumor challenge, while all three control mice developed tumors. One control mouse had delayed tumor growth.

for prevention as well as therapeutic studies. Mammary tumors, which can be followed by palpation, are useful for therapeutic studies, as tumor location alleviates the need to sacrifice the animal to determine clinical response and is optimal for prevention therapies, as the tumors develop after birth, and immunizations could precede tumor development. MUC1 is overexpressed in these tumors and immunization with a MUC1-specific vaccine significantly increases MUC1 serum levels and elicits a MUC1-specific cellular and a humoral immune response. Similar to humans, these immune responses do not translate into an antitumor response suggesting that a) the tumor cells successfully evade the immune effector cells using a variety of mechanisms, and b) targeting a single tumor antigen may not be effective. Similar to our study, recent studies showed that the HER-2/neu transgenic mice that are immunologically tolerant to the neu antigen and develop spontaneous unifocal mammary carcinomas, developed neu-specific T cells after vaccination. However, the neu-specific effector T cells were unable to protect these mice from the developing breast tumors (34,35).

MMTV-driven polyoma middle T antigen affects almost every cell in the mammary gland, and the entire mammary tree is burdened with tumors, which may be too aggressive for the immune effector cells to act effectively. We have previously shown by adoptive transfer that MUC1-specific CTL developed in vitro can eradicate less aggressive, transplantable MUC1$^+$ tumors (4). Whether these CTLs gain access into the growing tumor mass was tested by adoptively transferring carboxyfluorescein succinimidyl ester-labeled MUC1-specific CTL into tumor-MMT mice. Preliminary data suggest that CTLs can enter the tumor mass and multiply for up to 14 days, after which they are undetectable in the tumor mass (unpublished data).

Many reports have suggested that progressing tumors in patients with cancer have elaborate means of escaping an apparently effective MHC class I restricted immune response (36,37). Other investigators have found that the CTL response occurs too late to be effective against the tumors (38). Tumors evade host immunity at both the induction and effector phases. Because immunized MMT mice have developed MUC1-specific CTL responses, these spontaneously arising mammary gland tumors must have evaded the existing CTL. We have shown that the immunosuppressive factor, TGF-β2 is expressed by MMT tumor cells and may be a factor responsible for rendering the CTLs cytolytically anergic (Fig. 9). It has been shown previously that TGF-β may alter TCR subcomponent composition and downregulate CD3ζ, γ, δ, but not CD3ε, thereby reducing T cell signaling and CTL responses against tumor cells, and reducing TGF-β2 expression reverses this effect (39). However, including TGF-β2 antibody treatment in our vaccination strategy did not lead to effective antitumor response in the MMT mice (unpublished data), suggesting that multiple immune evasion mechanisms may be used by the tumors to avoid immune destruction. We have demonstrated that the MMT tumor cells may avoid immune recognition by down-regulating MHC class I expression (Table 3). It has been recently shown that functionally active inhibitory receptors that impart negative signals have been found on T cells. One such inhibitory receptor, CD94-NKG2A, can induce cytolytic anergy in CTLs specific for polyomavirus induced tumors (40). Since the tumors in the MMT mice are induced by the polyomavirus middle T antigen, it is likely that a similar mechanism is occurring these mice. In our preliminary data, we observe that the tumor-infiltrated lymphocytes isolated from MMT tumors do not proliferate in response to MUC1 antigen nor are they cytolytic against MUC1-expressing tumor cells in vitro, suggesting that the effector T cells may be tolerant to the antigen or may be anergic (unpublished data). Further experiments need to be conducted to confirm these results.

The beneficial effect of IL-2 treatment may be attributed to stimulation of other tumor antigen-specific CTLs that may exist in these mice, but have never been identified in vitro. Analyzing some of the other known tumor antigen-specific CTLs may provide us with better answers and these studies are under way. We show that immunotherapy that targets multiple tumor antigens elicits an effective antitumor response, albeit in an injectable tumor model (Fig. 10). Similar studies are under way in the MMT mice.

Finally, targeting a single tumor antigen, such as MUC1, may lead to activation-induced tolerance and anergy of CTLs in vivo, which eventually results in inactivation of the effector T cells and interruption of an antitumor response. A defining feature for a tolerant T cell is its hyporesponsiveness to antigen when compared with either a naïve or a primed T cell, and experiments are under way to evaluate if MUC1-specific immunization leads to T cell tolerance. This phenomenon of antigen-specific CTL tolerance after peptide immunization is not new and has been reported by several investigators (38,41,42). Thus, immunotherapy must be effective in breaking the existing tolerance. Recent studies have highlighted the role of two costimulatory molecules, OX40 expressed on activated T helper cells and CD40 expressed on antigen presenting cells, as targets for therapeutic intervention in cancer. Both molecules belong to the tumor necrosis factor receptor family and are

implicated in preventing tolerance induction as well as reversing established tolerance observed during anticancer immunotherapy (43,44). These studies indicate that ligation of these costimulatory molecules with specific antibodies may be a useful strategy for enhancing T cell unresponsiveness to anticancer vaccination (45).

As is frequently observed with human immunotherapy clinical trials, there is a poor correlation between the ability to induce an in vitro cellular T cell response and a clinical response (46,47). The MMT model appropriately mimics the human condition and is an excellent model for testing therapy in a setting relevant to the treatment of patients with cancer as well as for prevention and delineation of the mechanisms of tolerance, immunity, and autoimmunity. This study has only touched upon some of the major issues that are critical for designing suitable immune therapies for cancer. Future immunizations in MMT mice will target multiple antigens and costimulatory molecules.

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Reduced T Cell and Dendritic Cell Function is Related to COX-2 Over-expression and PGE_2 Secretion in Patients with Breast Cancer

Barbara A. Pockaj, M.D.[*], Gargi D. Basu, Ph.D[+], Latha B. Pathangey[+], Richard J. Gray, M.D.[*], Jose L. Hernandez[+^], Sandra J. Gendler, Ph.D.[+] and Pinku Mukherjee, Ph.D.[+]

[*]Department of Surgery, [+]Department of Biochemistry and Molecular Biology, and [^]Department of Biostatistics, Mayo Clinic Scottsdale, 13400 E. Shea Blvd., Scottsdale, AZ 85259

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Direct Correspondence to:

Barbara A. Pockaj, M.D.
Department of Surgery
Mayo Clinic Scottsdale
13400 E. Shea Blvd.
Scottsdale, AZ 85259
(480) 301-6551
(480) 301-8414 (Fax)
pockaj.barbara@mayo.edu

Pinku Mukherjee, Ph.D.
Department of Biochemistry and Molecular Biology
Mayo Clinic Scottsdale
13400 E. Shea Blvd.
Scottsdale, AZ 85259
(480) 301-6327
(480) 301-7017
mukherjee.pinku@mayo.edu

Mini-Abstract

T cell and dendritic cell (DC) function were found to be impaired in breast cancer patients. In addition, we found cyclooxygenase-2 (COX-2) over-expression and elevated prostaglandin E_2 (PGE_2) levels in breast cancer. Data suggest a direct correlation between increased COX-2 and PGE_2 expression and impaired immune cell function.

Abstract

Background: In several neoplastic diseases, including breast cancer, immunosuppression correlates with disease stage, progression and outcome. Thus, thorough analysis of immune parameters in breast cancer patients may be beneficial in designing effective anti-cancer immune-based therapies.

Methods: We investigated dendritic cell (DC) and T cell function from breast cancer patients at various stages of the disease and age-matched controls. We also evaluated cyclooxygenase-2 (COX-2) expression and prostaglandin E2 (PGE_2) levels within the tumor milieu and in the circulation.

Results: T cells from cancer patients showed decreased proliferation in response to CD3 antibody stimulation. Analysis of Th1/Th2 cytokines revealed reduced levels of IFN-γ, TNF-α, IL-12 and IL-2 and increased levels of IL-10 and IL-4. DCs from these patients showed significantly reduced expression of co-stimulatory molecules (B7 and CD40) and demonstrated reduced phagocytic ability, reduced antigen presentation to T cells, and reduced ability to mature in response to lipopolysaccharide (LPS). Data revealed increased synthesis of PGE_2, an immune suppressor, along with increased expression of COX-2, a key regulator of PGE_2 synthesis.

Conclusions: COX-2-induced PGE_2 may contribute to the immunosuppression and may directly block anti-tumor immunity while promoting tumor growth, providing us with the rationale for using COX-2 inhibition combined with immunotherapy.

Introduction

The defective function of the host's immune system is one of the major mechanisms by which tumors escape from immune surveillance. T cell anergy is thought to be an early event in tumor progression and may precede the generalized immunosuppression that is observed in cancer patients [1]. Multiple mechanisms of tumor specific evasion have been demonstrated including impaired HLA expression, modulation in surface antigens, lack of co-stimulatory molecules on dendritic cells (DCs) leading to impaired antigen presentation to T cells, impaired T cell receptor (TCR) signal transduction through the TCR-ζ chain, and elaboration of immune-suppressive cytokines by tumor cells and T regulatory cells such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF-β) [2].

Functional impairment of T cells has been well documented in patients with cancer [3]. This is true of both circulating and infiltrating lymphocytes in which there is reduced activation of the T cell receptor and impaired production of interferon-gamma (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-alpha (TNF-α) in response to CD3 monoclonal antibodies [4]. Cytokine profiles of cancer patients demonstrate an abnormal balance between T cell helper-1 (Th1) and T cell helper–2 (Th2) cytokines favoring a Th2 response [5]. Circulating and tumor infiltrating DCs have also been shown to be functionally impaired. In metastatic melanoma patients, tumor infiltrating DCs express low levels of co-stimulatory molecules (CD80 and CD86) and therefore are unable to activate T cells, while DCs isolated from breast cancer patients demonstrate significantly reduced ability to stimulate allogeneic and antigen-specific T cell responses [6, 7]. In certain cancers, DCs derived from peripheral blood are lower in absolute number as compared to non-cancer individuals and are predominantly immature in phenotype [8].

Cyclooxygenase-2 (COX-2) is over-expressed in a variety of cancers including breast cancer [9-11]. COX-2 is an enzyme that converts arachidonic acid to prostaglandin H_2 that is further metabolized to other prostaglandins including prostaglandin E_2 (PGE_2) [12]. COX-2 expression is rapidly induced secondary to a number of factors including growth factors, tumor promoters, and hormones [13]. Transgenic mouse models demonstrated that over-expression of COX-2 leads to the development of mammary tumors [14]. Over-expression of COX-2 is also known to inhibit apoptosis [15] and promote angiogenesis [16]. This over-expression of COX-2 can lead to increased production of prostaglandins such as prostaglandin E_2 (PGE_2), which has multiple down stream effects. PGE_2 is known to transactivate the epidermal growth factor receptor (EGFR) that triggers mitogenic signaling in epithelial cells and induces cancer cell proliferation [17]. PGE_2 also causes immunosuppression *in vitro* [18] and it can induce immunosuppression *in vivo*, enhancing tumor growth in animal models [19, 20]. In this study we tested the hypothesis that the COX-2-induced PGE_2 overexpression may correlate with the global immunosuppression observed in breast cancer patients.

Since T cells and DCs are pivotal in the development of anti-tumor immunity and are susceptible to tumor-mediated immune-suppression, we investigated DC and T cell function from twenty-three breast cancer patients at various stages of the disease and compared the data to eight normal age-matched donors. Although there have been several studies describing the functional impairment of T cells and DC in breast cancer patients, the studies have not evaluated both T cell and DC function from the same breast cancer patients. Moreover, the mechanisms driving the functional impairment still remain elusive. The goal of our study was to evaluate the immune

status of patients presenting with the diagnosis of breast cancer and to evaluate the immune-modulating factors within the tumor milieu that may potentially account for the functional impairment of immune effector cells. This is the first study describing a thorough analysis of both T cell and DC function in newly diagnosed breast cancer patients. Impaired functionality of T cells and DCs correlated with COX-2 and PGE_2 over-expression. These studies are of critical importance for designing novel immunotherapeutic strategies for breast cancer but also in selecting the patients that may most benefit from such therapies.

Methods

Study Characteristics This research study was approved by the Mayo Clinic Institutional Review Board. Patients who presented to the Mayo Clinic Scottsdale Breast Clinic for initial treatment of disease were eligible for the protocol. The patients signed informed consents for peripheral blood and tumor samples. Informed consent for peripheral blood samples was also obtained from healthy, age-matched volunteers. A total of twenty-three patients with breast cancer and eight healthy controls were studied. Breast cancer patient demographics and tumor characteristics are shown in Table 1. Nearly all patients were post-menopausal with a mean age of 69 years. Normal donors were all post-menopausal with a mean age of 60 years. Infiltrating ductal adenocarcinoma was the most common tumor subtype (57%). The mean size of the tumors was 2.4 cm, although 71% of lesions were 2 cm or less. Only 20% of patients had lymph node metastases and the majority of patients presented with either stage I or stage II disease. In most cases, blood was drawn on the day of surgery, prior to resection of the tumor. In some cases, blood was drawn few days prior to surgery.

The overall schematic for the study design is shown in Figure 1. Whole blood was obtained from the study subjects and the peripheral blood mononuclear cells (PBMC) were separated using a Ficoll-Paque density gradient centrifugation. Sera from these patients were also collected and stored in –80°C freezer. The PBMC were used for isolation of T cells and DCs and the serum was used for cytokine/chemokine and PGE_2 evaluation. Surgically resected tumor tissue and lymph node metastasis were used to make tissue lysate for COX-2 and PGE_2 evaluation.

T Cell Isolation T cells were isolated from PBMC of patients as described in the schematic (Figure 1). Briefly, mononuclear cells were obtained by centrifugation of peripheral blood over Ficoll-Paque gradient (Amersham Biosciences, Sweden). Mononuclear cells were incubated for 2h at 37°C and non-adherent lymphocytes were removed and used as T cells and the adherent cells were used for generation of DCs.

T Cell Proliferation Assay Non-adherent lymphocyte population (1×10^6/ml) was subjected to *in vitro* stimulation with various concentrations of purified plate-bound CD3 antibody (BD Pharmingen, San Diego, CA). Cells were incubated for 4 days with CD3 antibody and ^{3}H-thymidine was added 24 hours prior to harvest. After washing off excess thymidine, cells were lysed with 5% Triton X-100, and incorporated thymidine was evaluated using the Topcount micro-scintillation counter (Packard Biosciences, Shelton, CT). Evaluation of T cell proliferation was also performed with varying concentrations of tumor lysates (12ug/ml-200ug/ml) and purified PGE_2 (Cayman Pharmaceutical, Ann Arbor, MI).

Analysis of Intracellular Cytokines Intracellular cytokine levels were evaluated by two-color flow cytometric analysis following TCR ligation. Intracellular cytokines were determined post brefeldin-A (BD Pharmingen) according to manufacturer's recommendation (4uls/1.2 X 10^7 cells/6mls for 3h at 37°C prior to staining). This treatment stops the release of cytokines in the culture media and the cytokines accumulate within the cells. Cells were then stained for surface markers for T cells (CD3) or DC (HLA-DR) at 4°C for 15 minutes followed by washing excess stain and permeabilizating cells with Pharmingen permeabilization solution (containing saponin) for 30 minutes at 4°C. Cells were then stained for intracellular IL-2, IL-12, IFN-γ, IL-4, IL-10, and TNF-α for 30 minutes at 4°C. Cells were analyzed using the Becton Dickinson FACScan and data analyzed using the Cell Quest Program. All antibodies were purchased from BD Pharmingen.

Serum Analysis of Cytokines and Chemokines A cytokine/chemokine array kit (Ray Biotech Inc., Norcross, GA) was used to detect a panel of 22 secreted cytokines and chemokines in the serum from normal and breast cancer patients. Manufacturer's recommended protocol was utilized.

DC Isolation and Maturation DCs were generated from $CD14^+$ monocyte population isolated from PBMC. Briefly, mononuclear cells were obtained by centrifugation of peripheral blood over Ficoll-Paque gradient. Mononuclear cells were incubated for 2h at 37°C and non-adherent cells were removed. Adherent cells were incubated with GM-CSF (5ng/ml, Pepro Tech, Rocky Hill. NJ.) and IL-4 (5ng/ml, PeproTech) for 4 - 5 days. Cells were collected, counted, and phenotyped for immature DC and further cultured for one additional day with GM-CSF

(5ng/ml), IL-4 (5ng/ml) and LPS (100ng/ml, Sigma Pharmaceuticals, St. Louis, MO). Cells were collected on day 6 as mature DC.

DC Phenotype Control and breast cancer patient DCs were analyzed by two-color flow cytometric analysis. Cell surface expression of several markers was evaluated: CD80 (B7-1), CD86 (B7-2), CD40, HLA-DR, HLA-ABC, CD1a and CD14. All antibodies were purchased from BD Pharmingen. Stained cells were analyzed utilizing the Cell Quest program on a Becton Dickinson FACScan.

DC Function A) Mixed Lymphocyte Reaction (MLR) Assay. Control and patient-derived DCs were assayed for their ability to stimulate allogeneic T cells in an MLR. T cells (1×10^5) from normal donors were incubated with irradiated DCs (3000 rads, 1×10^4 cells) from allogeneic breast cancer patients for a period of 5 days and ^{3}H-thymidine was added 24 hours prior to harvesting the cells. After washing off the excess thymidine, cells were lysed with 5% Triton X-100, and incorporated thymidine was evaluated using the Topcount micro-scintillation counter.

B) Phagocytosis ability. Immature and LPS-matured DCs from normal donors and breast cancer patients were incubated with FITC-conjugated dextran beads (MW: 40,000, Molecular Probes Inc., Eugene, OR) at 1 mg/1×10^6 cells for 30 minutes at 37^0 C. Dextran beads were used as the exogenous antigen source. Since the beads were conjugated to FITC, uptake of dextran beads by DCs was analyzed by flow cytometry and mean fluorescence intensity was calculated.

Breast Tumor Cell Lysates Tissue lysates were prepared within 1 hour post surgery by homogenizing the tumor tissue in lysate buffer containing 20 mM Hepes, 0.15M NaCL, and 1%

Triton X-100 supplemented with phosphatase inhibitor cocktail mix (1:100 dilution, Sigma Pharmaceuticals) and complete protease inhibitors (Roche Pharmaceuticals, Indianapolis, IN). Lysates were stored at -80°C freezer for further use.

Expression of COX-2 Protein in Tumor Lysate Protein concentrations of the lysates were determined by Pierce BCA protein assay kit (Pierce, Rockford, IL). SDS-PAGE electrophoresis was performed using 12% resolving gel. 100 μgs of protein was loaded per lane. Gels were immunoblotted and probed for COX-2 with specific COX-2 monoclonal antibody (goat polyclonal, clone C20, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 1:200 dilution.

PGE_2 in Serum and Tumor Lysate Levels in the lysates were determined using a specific ELISA kit for PGE_2 and levels in the serum were determined using the PGE_2 metabolite ELISA kit (Cayman Pharmaceuticals, Ann Arbor, MI). Manufacturer's recommended protocols were followed.

Statistical Analysis Statistical significance was assessed using pair-wise comparisons with the Tukey-Kramer adjustment for multiple comparisons. The margins-of-error for the comparisons were obtained by calculating the 95% confidence intervals for the differences between group proportions.

Immune function parameters were compared between a set of clinical indicators. The clinical parameters examined were stage, lymph node status, estrogen receptor status, tumor size (<2 cm vs. 2 cm), grade, presence of angiolymphatic invasion, multifocality, and history of previous

breast cancer. Due to the non-normality of the immune function data and the small sample size of the cohort, the exact Wilcoxon statistic was used in assessing significant differences between the groups. All error bars in the figures represent the standard deviation of the mean.

Results

The proliferative ability of T cells isolated from breast cancer patients just prior to surgical resection of tumor was examined. T cells were stimulated by various concentrations of plate-bound CD3 antibody and T cell proliferation measured by ^{3}H-thymidine uptake. T cell proliferation was significantly reduced in cancer patients compared to normal controls ($p<0.001$ at 1ug and 0.5ug/ml CD3 antibody) (Figure 2). Additional analysis of Th1/Th2 cytokines in activated cells revealed reduced intracellular levels of the immunostimulatory Th1 cytokines IFN-γ ($p<0.001$), TNF-α ($p<0.001$), IL-12 ($p=0.002$) and IL-2 ($p<0.001$), and increased levels of Th2 cytokines, IL-4 ($p=0.02$) and IL-10 ($p=0.003$), Figure 3.

DCs from patients with breast cancer expressed reduced levels of co-stimulatory molecules such as CD80 (B7.1), CD86 (B7.2), and CD 40 upon maturation with LPS (Figure 4). Similar results were obtained with TNF-α-induced maturation. Other markers utilized to determine DC phenotype included HLA-DR, HLA-ABC, CD1a and CD14 (data not shown). No differences between cancer patient and normal donors were observed in these markers. The reductions in expression of both CD80 and CD86 were statistically significant, $p<0.001$. This low expression of co-stimulatory molecules is an indication that these DCs remain immature. It has been suggested that immature DCs possess reduced ability for stimulating T cells and therefore may contribute to tumor-induced T cell tolerance rather than immunity, and that mature DCs are

essential for presenting tumor antigens and activating T cells to become cytolytic against tumor cells.

We therefore tested if DCs from these cancer patients had reduced antigen presentation. Data indicate that the function of DCs was significantly reduced. DCs from breast cancer patients demonstrated significantly reduced ability to present antigen to allogeneic normal T cells in a Mixed Lymphocyte Reaction (MLR), $p<0.001$ (Figure 5A). The MLR results support the previous findings shown in Figure 4 where the same DCs expressed low levels of co-stimulatory molecules and therefore failed to fully mature. Furthermore, the immature DCs from breast cancer patients demonstrated significantly reduced ability to phagocytose exogenous antigens *in vitro*, $p<0.002$ (Figures 5B and C). They also demonstrated a maturation defect when stimulated with LPS treatment (Figures 5C). Figure 5B shows a representative histogram from one patient and Figure 5C shows a bar graph of average values from the 23 patients. Immature DCs are known to be strong phagocytes but weak antigen presenters whereas mature DCs are weak phagocytes and strong antigen presenters [21]. We found that immature DCs from normal donors showed significantly higher ($p<0.002$) fluorescence intensity (mean intensity of 1620) demonstrating good phagocytic ability and upon maturation with LPS, their phagocytic activity was reduced (mean intensity of 265) (Figure 5C). In comparison, the immature DCs from the breast cancer patients had significantly lower fluorescence intensity (mean intensity of 270) indicating poor phagocytosis and the florescence intensity of these DCs did not decrease with LPS treatment (mean 220), once again indicating impaired maturation (Figure 5C). Similar results were obtained when TNF-α instead of LPS was used to mature DCs (data not shown).

Tumor cells secrete factors that are known to induce immunosuppression and promote tumor cell proliferation. Prostaglandins, especially PGE_2, is one such factor expressed within the tumor microenvironment as well as secreted in the serum. Since COX-2 is the key regulator of prostaglandin synthesis, we evaluated the COX-2 protein expression on western blots of adjacent normal tissue lysates, tumor tissue lysates and lysates from lymph nodes that contained metastases. The COX-2 protein was over-expressed in both the tumor and lymph node metastases compared with normal tissue, with the highest expression being observed in lymph node metastases (Figure 6A). Next, we evaluated the amount of PGE_2 in the serum of breast cancer patients and normal donors by specific PGE_2 metabolite ELISA. Since PGE_2 is rapidly converted in the serum by 15-OH PGDH to its 13,14-dihydro-15-keto metabolite, direct measurement of intact PGE_2 is not possible in sera or plasma. Thus PGE_2 metabolite measurement is necessary to provide a reliable estimate of actual PGE_2 production [22, 23]. However, in the tumor tissue lysates, PGE_2 levels can be evaluated directly using a specific ELISA for PGE_2 as these cell types do not contain the enzymes required for metabolism of PGE_2, thus keeping the PGE_2 levels stable. Breast cancer patients had significantly elevated levels of PGE_2 metabolite levels in their sera as compared to normal, $p<0.001$ (Figure 6C). Similar to COX-2 expression, we observed high levels of PGE_2 in the tumor cell lysates ($p=0.042$) and lymph node metastases ($p=0.017$) as compared to normal adjacent breast tissue (Figure 6B).

Since tumor lysates contained COX-2 and PGE_2 and presumably other immunosuppressive factors, we determined if these lysates could directly block T cell signal transduction and activation that lead to T cell proliferation. We evaluated the effect of the tumor lysates on the

proliferation of T cells from the normal donors. We used purified PGE_2 as standard and compared it to the inhibition observed with the tumor lysate. There was direct inhibition of normal T cell proliferation in response to CD3 antibody by the tumor lysates and by purified PGE_2 (Figure 7). The inhibition observed with tumor lysate was higher than purified PGE_2, suggesting the presence of other T cell inhibitory agents in the tumor lysate.

We evaluated our data to see whether the patient's clinical variables correlated with immune function parameters. The clinical parameters examined were stage, lymph node status, estrogen receptor status, tumor size ($\leq$2 cm vs. 2 cm), grade, presence of angiolymphatic invasion, multifocality, and history of previous breast cancer. No differences in T cell function or serum PGE_2 were noted among these groups. Dendritic cell immaturity and phagocytic ability were significantly higher ($p<0.05$) in patients with higher stage and patients with lymph node metastases. There were no significant differences among the other clinical parameters, which could be due to small sample size.

Discussion

There is evidence that tumor-specific antigens are present on cancer cells that could function as potential targets for the immune system. Unfortunately, cancer patients fail to mount an effective immune response against them, indicating that the immune cells are tolerant to the tumor-specific antigens. Breaking this tolerance is one of the major goals of immunotherapy for cancer. Tumors also exhibit multiple immunosuppressive strategies, such as down regulation of MHC class I molecules, lack of co-stimulatory molecules on DCs and secretion of immunosuppressive cytokines as well as production of high levels of COX-2 and PGE_2. We first examined the

immune status of patients recently diagnosed with breast cancer and then evaluated the impact of COX-2 over-expression by the tumor cells and subsequent synthesis of PGE_2 on the tumor's ability to evade immune surveillance.

Overall we found significant functional impairment in the T cells of patients who were diagnosed with breast cancer. T cells isolated from breast cancer patients prior to surgical removal of the tumor demonstrated marked reduction in their proliferation response to CD3 antibody (Figure 2), suggesting a defect in activation of the TCR mediated signal transduction pathways [24]. These described defects include reduced TCR-ζ chain expression, a defect in transcription factors such as nuclear factor-κ-B, up regulation of cyclin-dependent kinase inhibitor p27kip1, and hydrogen peroxide production by activated granulocytes [24-28]. A likely consequence of this ineffective T cell signaling is impaired cytokine production by the T cells [5, 29]. Nieland found that reduced cytokine expression was found in early breast cancer patients with a normal TCR-ζ chain, thus suggesting that another mechanism may be responsible [29].

Th1 cytokines promote the development of cell-mediated anti-tumor response [30]. On the other hand, Th2 cytokines are necessary for humoral immunity. Patients with carcinoma have a predominance of Th2 cytokines in the peripheral blood [5]. We found a similar increase in Th2 cytokines (IL-10 and IL-4) in our breast cancer cohort when compared to controls and reduced Th1 cytokines (IFN-γ, IL-2, and IL-12) (Figure 3). A shift to a Th2 response has been correlated with increasing stage in patients with renal cell carcinoma [31]. Preliminary analysis of serum chemokines and cytokines levels using a cytokine array system revealed a correlation between increasing levels of certain cytokines and chemokines with advanced stage breast cancer (Figure

8). Rantes, monokine induced by IFN-γ (MIG), monocyte chemoattractant protein-1 (MCP-1), IL-8 and IL-10 levels (proteins that favor a Th2 response) were higher in the sera from patients with high-grade tumor and lymph node metastases as compared to patients with low-grade tumor and no lymph node metastases. MCP-1 is implicated in tumor cell migration and invasion and in multi drug resistance [32]. Similarly, MIG and Rantes favor inflammation and tumor cell proliferation and invasion [33, 34]. IL-8 is implicated in increased angiogenesis and multi drug resistance [32] and IL-10 is known to cause T cell anergy [35]. TGF-β1 is known to downregulate both CTL and T helper signal transduction and function [36]. These chemokines and cytokines are released by monocytes, macrophages and lymphocytes that express the EP receptors for binding to PGE_2 [37]. It is therefore plausible that the high levels of PGE_2 observed within the tumor microenvironment and in the circulation may activate the tumor infiltrating lymphocytes, monocytes and macrophages via the EP receptors to release factors that favor tumor growth and invasion as well as suppress immune effector cells. These proteins were either absent or present in very low levels in the sera of normal controls. Conversely, serum levels of the immunostimulatory cytokines TNF-β1 and IFN-γ were higher in patients with low-grade/non-metastatic tumor when compared to patients with high-grade/metastatic tumor, once again implicating that advanced stage patients are more likely to be immunosuppressed. The cytokine/chemokine array analysis has been done on 6 breast cancer patients and 3 normal donors. Representative blots from two cancer patients and one normal donor are shown in Figure 8. The tumor milieu has been thought to play a significant role in the impairment of DCs. Release of such factors as IL-6, IL-10, M-CSF, vascular endothelial growth factor (VEGF), and macrophage inflammatory protein 3-alpha (MIP-3α) can prevent DC maturation and antigen presenting function. Interestingly, in our preliminary analysis of four breast cancer patient sera,

we observed increased levels of VEGF, M-CSF, IL-10 and MIP3-α (data not shown) as compared to normal. These results fit well with the concept that these elevated chemokine levels favor a Th2 response that limits the DC allo-stimulatory capability [7, 38-40]. In one study, it was shown that surgical removal of primary tumor resulted in a dramatic reduction in the proportion of immature DCs, although the levels never reached that of the normal controls [8].

Mature DCs are the most powerful antigen presenting cells and thus initiate the immune response [21]. The presence of immature DCs is thought to contribute to the induction of tolerance instead of immunity against the tumor antigens [41]. Low expression of co-stimulatory molecules on circulating DCs is an indication of immaturity. Low levels of co-stimulatory molecules in peripheral and draining lymph node DCs of breast cancer patients and impaired allostimulatory ability have been demonstrated in patients with breast cancer [7, 8]. Our study confirms some of these findings as shown by the low expression of co-stimulatory molecules on circulating DCs from our breast cancer patients (Figure 4). Our data further demonstrate the immaturity of DCs by the fact that DCs from cancer patients have reduced ability to present antigen to allogeneic normal T cells in a mixed lymphocyte reaction assay (Figure 5A).

Heightened endocytic activity is characteristic of cytokine-derived DCs and their enhanced capacity to capture and process antigens [21]. Our study demonstrated impaired phagocytosis by the immature DCs from breast cancer patients (Figure 5B and 5C). Attempts to maturate the DCs with LPS did not change their phagocytic ability, once again suggesting a defect in dendritic cell maturation.

What mechanisms underlie the T cell and DC anergy in cancer patients are unknown but probably involve multiple events. We evaluated if overexpression of COX-2 and downstream PGE_2 synthesis may be one of the mechanisms for immunosuppression. Interestingly, the COX-2 expression was high in primary tumors and even more prominent in lymph node metastases (Figure 6A). Since COX-2 was over-expressed, we evaluated the PGE_2 levels in the serum of the breast cancer patients and controls along with tumor lysates. Elevated levels of PGE_2 were demonstrated in both the sera and tumor lysates of patients with cancer (Figure 6B and 6C). PGE_2 is an immune suppressor, targeting both cytotoxic as well as helper T cell functions. PGE_2 is thought to suppress cell-mediated immune response while enhancing humoral immune response [42, 43]. PGE_2 suppresses chemokine and cytokine production in humans including IFN-γ, TNF-α, IL-12, and IL-1β mediated expression of chemokines. PGE_2 upregulates expression of immunosuppressive cytokines, such as IL-10 and TGF-β [43, 44]. This immunosuppressive effect of PGE_2 was demonstrated by inhibition of normal T cell proliferation to tumor lysates with high concentrations of PGE_2 (Figure 7).

The ability of mature DCs to act as potent antigen presenting cells is related to their production of IL-12 [45]. DCs deficient of IL-12 generated in the presence of PGE_2 promote a Th2 response [46]. A recent study demonstrated that high concentrations of PGE_2 caused decreased IL-12 production via increases in IL-10 production and therefore decreased dendritic cell function [18]. This correlates well with our data, which clearly demonstrate elevated PGE_2 levels in the serum and within the tumor milieu, as well as show increased levels of intracellular IL-10 and decreased levels of IL-12 in T cells of our breast cancer patient population.

Thus, tumor over-expression of COX-2 via the elaboration of PGE_2 and other mechanisms could directly block the patient's defense mechanism against cancer and promote breast cancer growth [47]. We observed over-expression of COX-2 and PGE_2 and impaired T cell and DC function in breast cancer patients. If the immune system of breast cancer patients were persistently compromised, the success of immunotherapies would be limited unless the immune system could be appropriately stimulated. Many immunotherapies for cancer treatment have been partially successful in eliciting a cellular immune response; however, this response has been down regulated by tumor-derived immunosuppressive factors. If mediators of immune-suppression such as COX-2 and PGE_2 can be reduced, and co-stimulation for CTL effector function can be provided with appropriate immune-based therapy to overcome the tolerizing effects of the tumor, and most importantly, if tumor cell proliferation can be restricted, then immunotherapy can be very effective. This study along with other studies in the literature provides us with an immunologic rationale for using COX-2 inhibition that would reduce the PGE_2 levels and therefore reduce immunosuppression as well as reduce tumor cell growth. COX-2 inhibition combined with immune-based therapy that would induce CTL activity against tumor cells is a novel concept that needs further exploration in preclinical animal models and in clinical settings.

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Figure Legends

Figure 1. Schematic representation of the study design.

Figure 2. T cells from breast cancer patients have markedly reduced proliferation in response to specific TCR ligation. T cell proliferation in response to plate-bound CD3 antibody comparing breast cancer patients (____ , n = 23) and normal controls (_ _ _ _ , n = 8). Amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. Error bars represent standard deviation of the mean.

Figure 3. Reduced Th1-type cytokine and increased Th2-type cytokine in peripheral blood of patients with breast cancer. Comparison intracellular cytokine levels by flow cytometry. IFN-γ, TNF-α, IL-12, IL-2, IL-10 and IL-4 levels were compared between breast cancer patients (solid bars, n = 7) and normal controls (open bars, n = 3). Error bars represent standard deviation of the mean.

Figure 4. DCs from patients with breast cancer expressed reduced levels of co-stimulatory molecules. Flow cytometric analysis of surface expression of co-stimulatory molecules, CD80 (B7.1), CD86 (B7.2), and CD40 on LPS-matured DCs of breast cancer patients (solid bars, n = 23) vs. normal controls (open bars, n = 8). Similar results were obtained with TNF-α-matured DCs (data not shown). Error bars represent standard deviation of the mean.

Figure 5. DCs from breast cancer patients demonstrate significantly reduced ability to present antigen to allogeneic normal T cells and demonstrates reduced phagocytosis of exogenous antigen. A) Allogeneic antigen presentation to normal T cells by DCs of breast cancer patients (◊ n = 23) vs. normal controls (▲ n = 8) in a Mixed Lymphocyte Reaction. Amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. B) Representative histogram of immature and LPS-matured dendritic cell phagocytic ability of a cancer patient versus normal donor (the numbers on the right corner are the mean fluorescence intensity) C) Dendritic cell phagocytic ability (mean fluorescence intensity) in breast cancer patients (solid bars, n = 23) vs. normal controls (open bars, n = 8) in mature and immature state. In 5B and C, mean fluorescence intensity is used as a measure for the amount of FITC-conjugated dextran beads engulfed by the DCs. Error bars represent standard deviation of the mean.

Figure 6. COX-2 and PGE2 is over-expressed in tumor tissue and serum of patients with breast cancer. A) COX-2 protein levels in tissue lysates (100ug) from adjacent normal breast tissue, breast tumors, and lymph node metastases were analyzed by western blot analysis. Brackets indicate tissues from a single patient. Six patient samples were analyzed. B) PGE_2 levels in tissue lysates from adjacent normal breast tissue, breast tumors, and lymph node metastases were determined by specific ELISA. C) Serum PGE_2 metabolite levels in breast cancer patients (solid bars) vs. normal controls (open bars) were analyzed by specific PGE_2 metabolite ELISA. Error bars represent standard deviation of the mean.

Figure 7. Direct inhibition of normal T cell proliferation by factors present in the tumor lysate. T cell proliferation was determined in response to plate-bound CD3 antibody in the presence or absence of tumor lysate or purified PGE_2 at varying concentrations (0ug/ml – 200ug/ml). T cells were generated from normal donors (n = 4). Amount of ^{3}H-thymidine uptake directly corresponds to the proliferative capacity of T cells. Tissue lysis buffer was used as the negative control and purified PGE_2 was used as the positive control.

Figure 8. Serum analysis of chemokine/cytokine array revealed a correlation between increasing levels of certain cytokines and chemokines with advanced stage breast cancer. Expression of a panel of 22 secreted cytokines and chemokines was detected in the serum of normal and breast cancer patients using the RayBiotec Cytokine Array kit. Sera from one normal control, one patient with a grade 1 invasive breast cancer without lymph node metastases, and one patient with a grade 3 invasive breast cancer with lymph node metastases are shown. Similar results were observed with the other five breast cancer patients tested. The boxes on the blots and table demonstrate the cytokines and chemokines that are either upregulated (+) or downregulated (-) compared to the normal serum. The open box represents the immunostimulatory cytokines; the gray box represents chemokines that favor aggressive tumor growth and the black box represents immunosuppressive cytokines. The actual cytokine array map from RayBiotec is also provided.

Table 1. Patient and Tumor Characteristics

Mean Age (Range)	69 years (36-80 years)
Pre-Menopausal	1 (4%)
History of Contralateral Breast Cancer	4 (17%)
Tumor Type	
DCIS	1 (4%)
Infiltrating Ductal	13 (57%)
Infiltrating Lobular	4 (17%)
Mixed Infiltrating Ductal/Lobular	2 (9%)
Infiltrating Mucinous	3 (13%)
Mean Tumor Size (Range)	2.4 cm (0.3-13 cm)
Tumor $\leq$ 2 cm	15 (71%)
Bloom Richard Grade	
1	8 (38%)
2	5 (24%)
3	8 (38%)
Estrogen Receptor Positive	19 (83%)
Lymph Node Metastases	5 (24%)
Number of Lymph Nodes Positive	1-8
Stage	
0	1 (4%)
1	10 (43%)
2	10 (43%)
3	1 (4%)
4	1 (4%)
5	1 (4%)

Figure 1

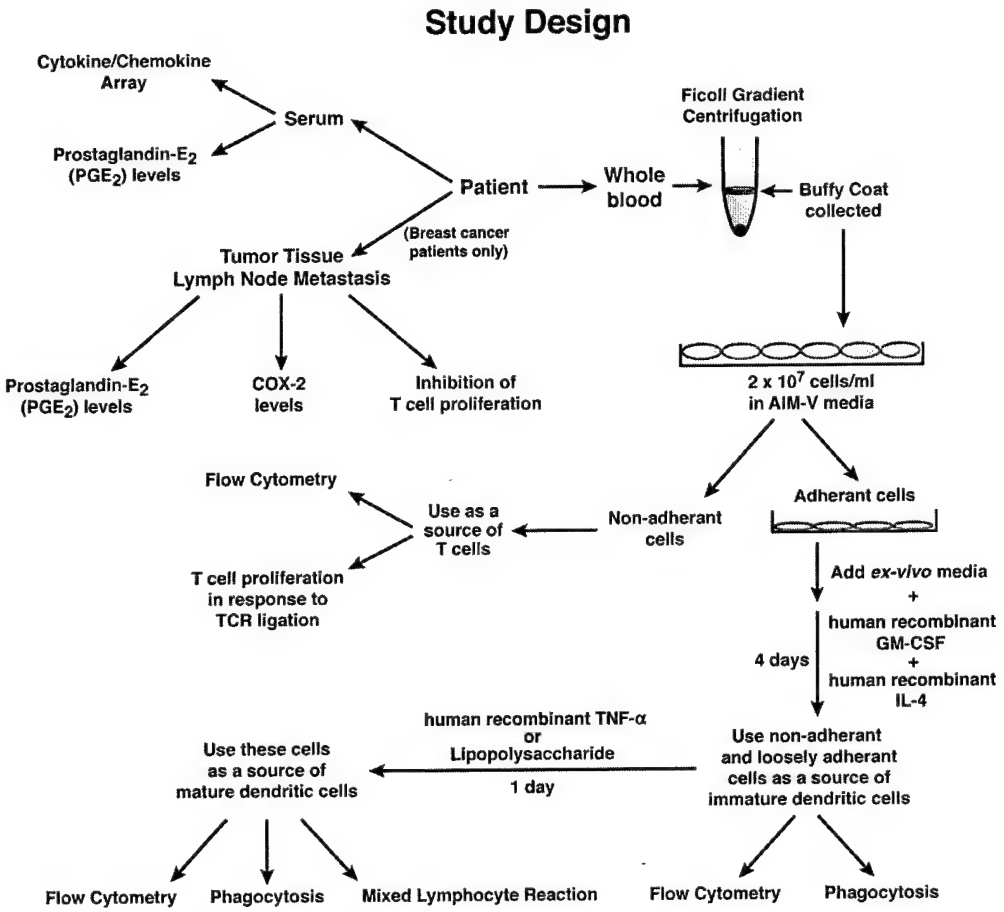

Study Design
Cytokine/Chemokine Array
Serum
Prostaglandin-E2 (PGE2) levels
Patient
(Breast cancer patients only)
Tumor Tissue Lymph Node Metastasis
Prostaglandin-E2 (PGE2) levels
COX-2 levels
Inhibition of T cell proliferation
Ficoll Gradient Centrifugation
Whole blood
Buffy Coat collected
2 x 10^7 cells/ml in AIM-V media
Flow Cytometry
Use as a source of T cells
T cell proliferation in response to TCR ligation
Non-adherant cells
Adherant cells
Add ex-vivo media
+
human recombinant GM-CSF
+
human recombinant IL-4
4 days
human recombinant TNF-α
or
Lipopolysaccharide
1 day
Use these cells as a source of mature dendritic cells
Use non-adherant and loosely adherant cells as a source of immature dendritic cells
Flow Cytometry
Phagocytosis
Mixed Lymphocyte Reaction
Flow Cytometry
Phagocytosis

Figure 2

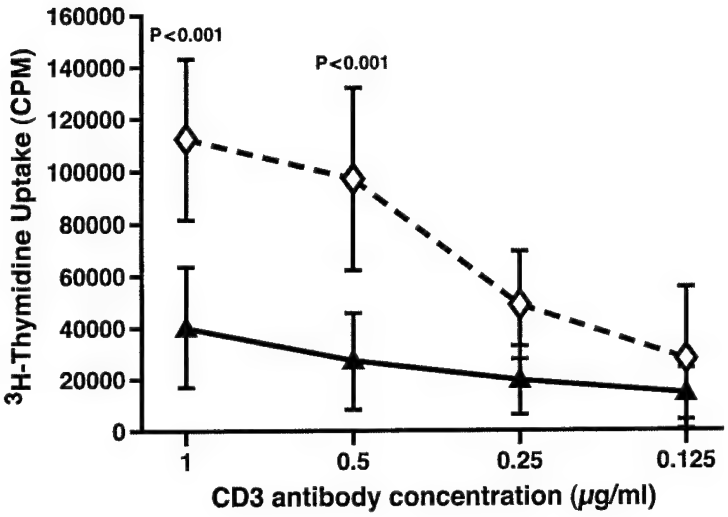

160000
140000
120000
100000
80000
60000
40000
20000
0
P<0.001
P<0.001
^{3}H-Thymidine Uptake (CPM)
1
0.5
0.25
0.125
CD3 antibody concentration (μg/ml)

Figure 3

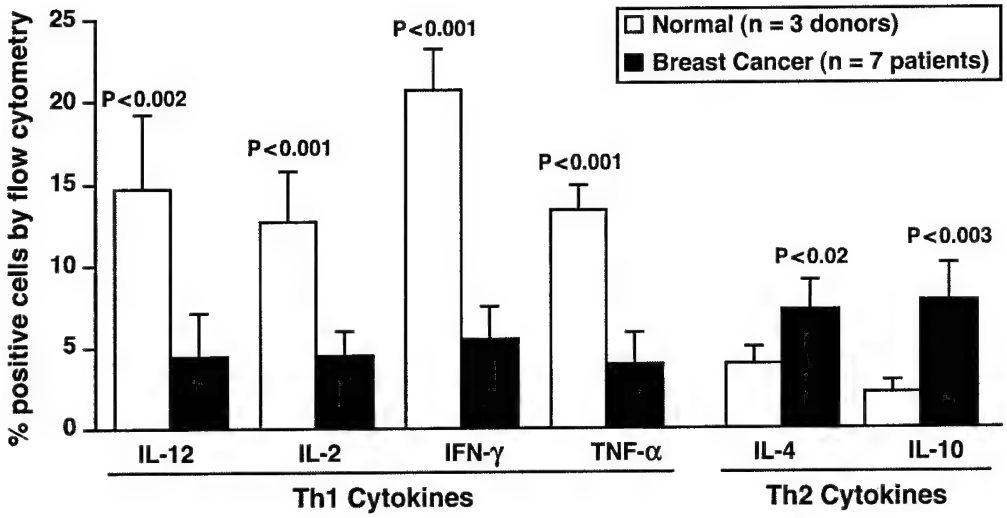

% positive cells by flow cytometry
25
20
15
10
5
0
Normal (n = 3 donors)
Breast Cancer (n = 7 patients)
P<0.002
P<0.001
P<0.001
P<0.001
P<0.02
P<0.003
IL-12
IL-2
IFN-γ
TNF-α
IL-4
IL-10
Th1 Cytokines
Th2 Cytokines

Figure 4

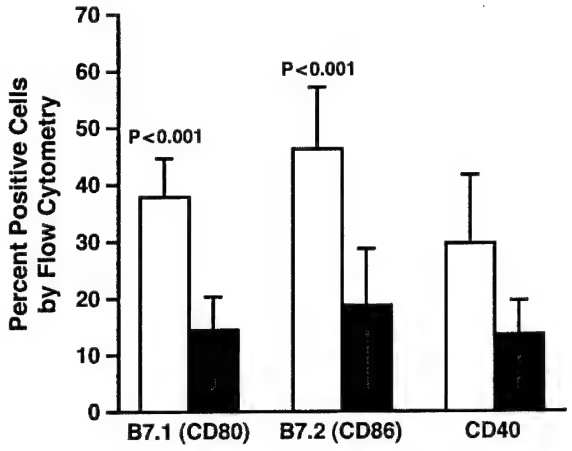

Percent Positive Cells by Flow Cytometry
70
60
50
40
30
20
10
0
P<0.001
P<0.001
B7.1 (CD80)
B7.2 (CD86)
CD40

Figure 5

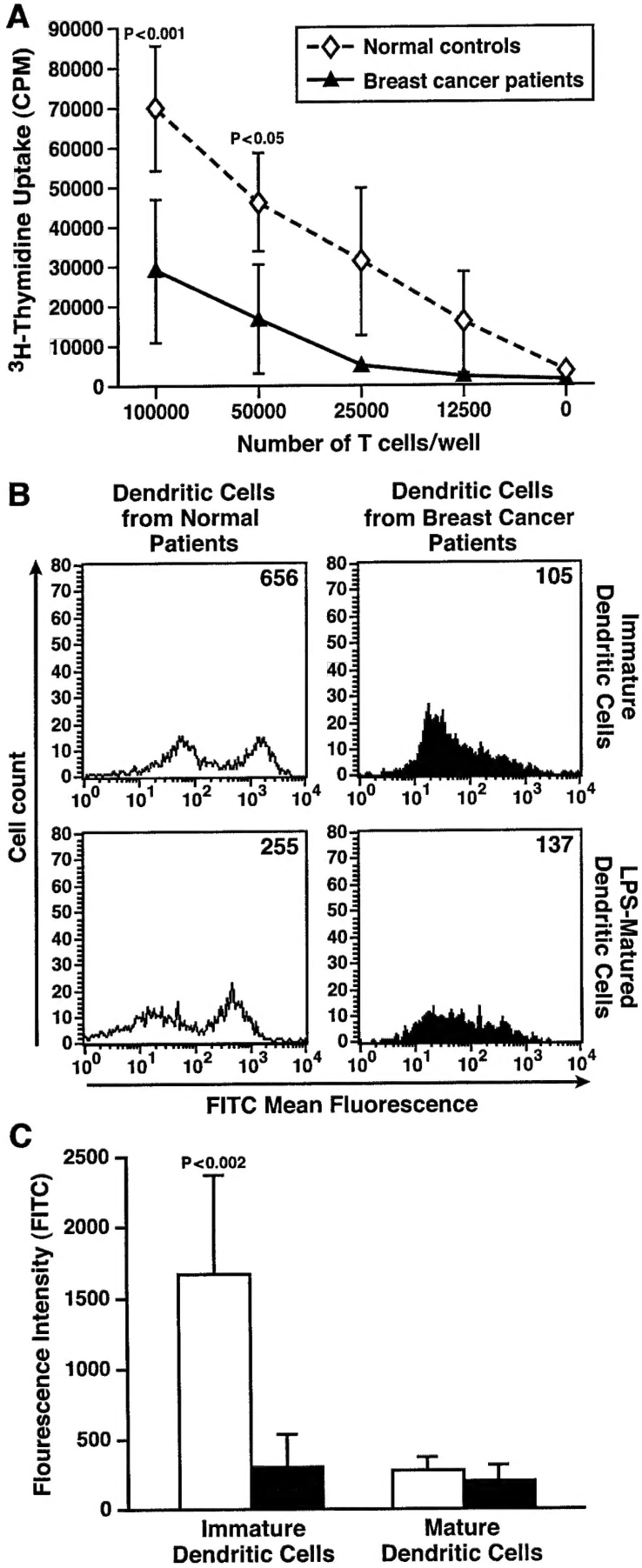

A
P<0.001
P<0.05
Normal controls
Breast cancer patients
^{3}H-Thymidine Uptake (CPM)
90000
80000
70000
60000
50000
40000
30000
20000
10000
0
100000
50000
25000
12500
0
Number of T cells/well
B
Dendritic Cells from Normal Patients
Dendritic Cells from Breast Cancer Patients
656
105
255
137
Immature Dendritic Cells
LPS-Matured Dendritic Cells
Cell count
FITC Mean Fluorescence
C
P<0.002
Flourescence Intensity (FITC)
2500
2000
1500
1000
500
0
Immature Dendritic Cells
Mature Dendritic Cells

Figure 6

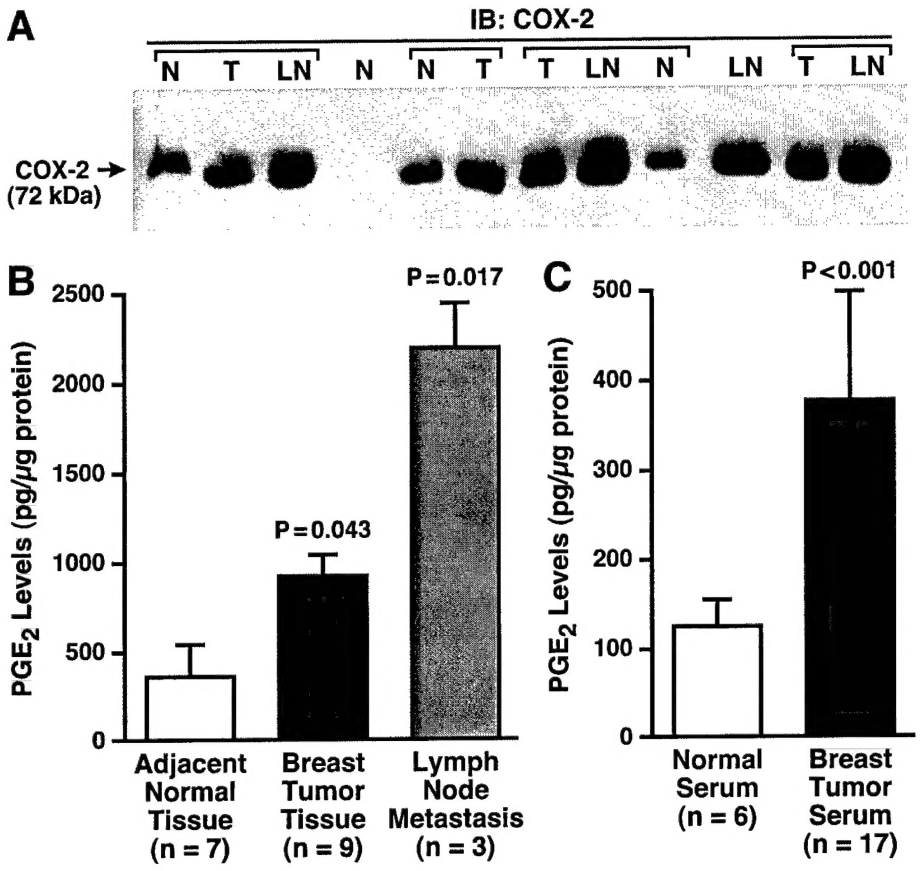

A
IB: COX-2
N T LN N N T T LN N LN T LN
COX-2 →
(72 kDa)
B
PGE2 Levels (pg/μg protein)
2500
2000
1500
1000
500
0
P=0.043
P=0.017
Adjacent Normal Tissue (n = 7)
Breast Tumor Tissue (n = 9)
Lymph Node Metastasis (n = 3)
C
PGE2 Levels (pg/μg protein)
500
400
300
200
100
0
P<0.001
Normal Serum (n = 6)
Breast Tumor Serum (n = 17)

Figure 7

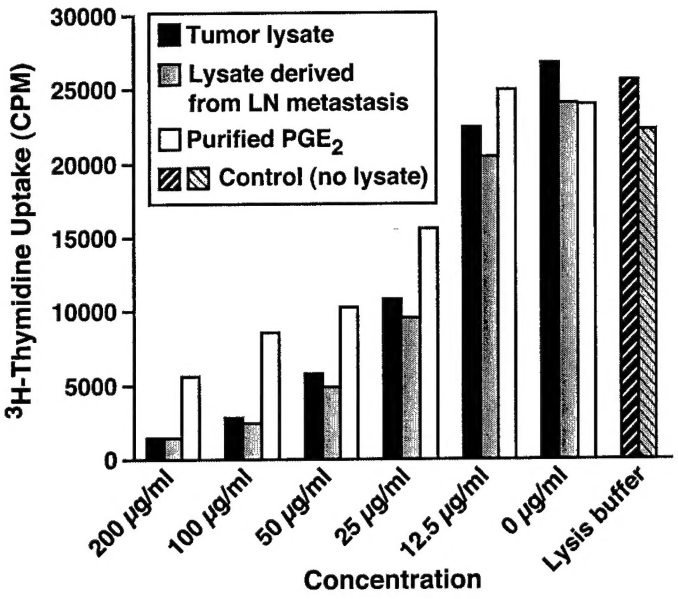
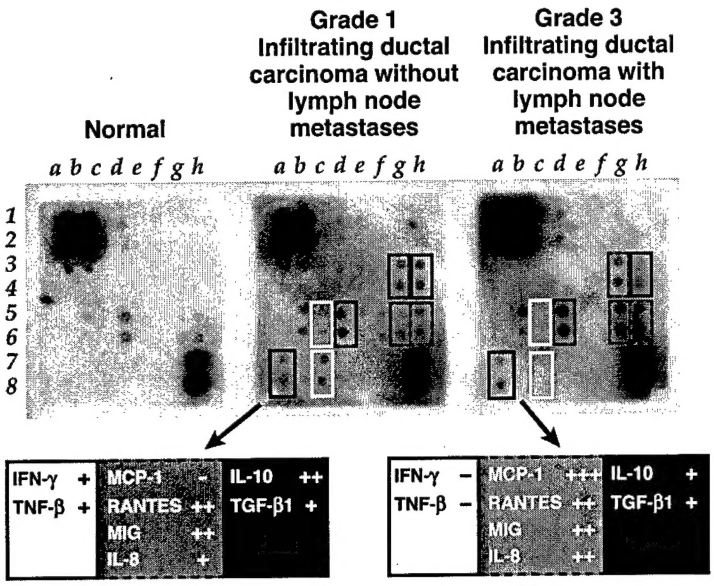

30000
25000
20000
15000
10000
5000
0
^{3}H-Thymidine Uptake (CPM)
Tumor lysate
Lysate derived from LN metastasis
Purified PGE_2
Control (no lysate)
200 µg/ml
100 µg/ml
50 µg/ml
25 µg/ml
12.5 µg/ml
0 µg/ml
Lysis buffer
Concentration

Figure 8



RayBio™ Human Cytokine Array Map

	a	*b*	*c*	*d*	*e*	*f*	*g*	*h*
Row 1 *Row 2*	Pos	Pos	Neg	Neg	GCSF	GM-CSF	GRO	GRO-α
Row 3 *Row 4*	IL-1α	IL-2	IL-3	IL-5	IL-6	IL-7	IL-8	IL-10
Row 5 *Row 6*	IL-13	IL-15	IFN-γ	MCP-1	MCP-2	MCP-3	MIG	RANTES
Row 7 *Row 8*	TGF-β1	TNF-α	TNF-β	Blank	Blank	Blank	Blank	Pos

Immuno-Stimulatory Cytokines

Chemokines that favor aggressive tumor growth

Immuno-Supressive Cytokines